1-1-1998

Genetic mapping and functional studies of a natural inhibitor of the insulin receptor tyrosine kinase: the mouse ortholog of human [alpha subscript 2]-HS glycoprotein

Vivian J. Cintron

GENETIC MAPPING AND FUNCTIONAL STUDIES OF A NATURAL INHIBITOR OF THE INSULIN RECEPTOR TYROSINE KINASE: THE MOUSE ORTHOLOG OF HUMAN α_2-HS GLYCOPROTEIN

by

VIVIAN J. CINTRON

DISSERTATION

Submitted to the Graduate School

of Wayne State University

Detroit, Michigan

in partial fullfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

1998

MAJOR: BIOLOGICAL SCIENCES
(MOLECULAR BIOLOGY)

Approved by:

10/5/98
Co-Advisor Date

10/4/98
Co-Advisor Date

Dedication

Dedicated to my beloved husband,

Antonio Chan "my Tony"

for his infinite love and support and to my parents,

Modesto Cintrón and Jesusa Roque

for giving me life.

Acknowledgments

It has been a pleasure to have worked with an incredible talented team in the Diabetes Research Laboratory of Dr. George Grunberger. In this laboratory long hours of work and effort were also compensated with friendship, humor and sincerity.

I remember when I approached Dr. Grunberger on February 1994, to work as a doctoral student in his laboratory. My desire was to engage in a research full of new techniques and new challenges and to make myself proud of my doctoral degree training.

Dr. George Grunberger believed in me as a student, and he gave me the opportunity to explore new ideas in the challenging field of insulin action. This was a new field for me when I started. Now close to my defense, I have learned that my project is just a small portion of the whole pathway and that many things still need to be discover and unravel. The mechanisms of insulin signaling is an extraordinary field that I would like to pursue with eagerness as my future career and I want to thank Dr. Grunberger for guiding me in this field, for being a wonderful advisor, and an excellent professor.

I want to thank Dr. Anton S. Goustin, for teaching me all the molecular biology techniques and also for giving me the opportunity to work in his laboratory for the most part of my dissertation. In his laboratory, I learned valuable points concerning molecular genetics providing me a great opportunity to develop new ideas for the project. Dr. Goustin have been a great professor and a great advisor throughout all these years. He has also been a great defender of my work and an excellent friend. Thank you Dr. Goustin for your time and effort.

While I was working in Dr. Goustin's laboratory, I met Dr. Robert A. Thomas. At the time he was a post-doctoral fellow and taught me many techinques in cloning and in

radiolabeling. Dr. Robert A Thomas dedicated his own time to promote my understanding in bacterial genetics and in computer analysis. But most of all, I have to thank him for his friendship. Dr. Robert A. Thomas was always there to give me support and encouragement through all these years. He was always there to give me a book to read, or to place an inspirational keepsake or picture over my desk. I should call him my "Angel", since he was always around when I needed him. Now, Dr. Thomas has his own laboratory and he is an Assistant Professor in the Center for Molecular Medicine and Genetics. I am very proud of him and also very secure that he will be an excellent mentor for his future students to come. I think that Dr. Thomas' patience to listen and care are his greatest legacy.

My special thanks go to Dr. Pothur R. Srinivas, who was also a research associate at Dr. Grunberger's laboratory when I started. In fact, Dr. Srinivas and Dr. Thomas were always watching over me and teaching me everyday techniques. As the time passed, Dr. Srinivas, (everyone calls him "PR"), also became an Assistant Professor in the Department of Internal Medicine at the School of Medicine. PR has been a "big brother" in the laboratory, and also a serious mentor. I learned from him everything related to the molecular biology of proteins. I will always remember when he asked me "Do you think only cloning is molecular biology, well, it is not, proteins can talk and discerning that pathway is molecular biology, too". I want to thank Dr. Srinivas for his valuable teaching and friendship.

I want to thank my colleague, Dr. Suresh Mathews, because he was always attentive and optimistic. His everyday sense of humor inspired me to keep going with my work and his competitive thinking also inspired me with new challenges for every Tuesday's laboratory meeting. I want to thank Dr. Mathews for patiently reviewing most

of my manuscripts and for his serious and wise critiques of my research work.

I want to direct special thanks to Mrs. Tessie Sharp for bringing me to Wayne State University and for making my education possible, and also to Dr. Dennis Smith and the late Dr. Hector Fernandez from the Department of Biological Sciences.

Special thanks are also dedicated to the members of my committee: Dr. Hiroshi Mizukami, Dr. Ann Sodja, Dr. David Njus at the Department of Biological Sciences for dedicating time and effort and providing me with support in the completion of my graduate program..

I would like to dedicate special thanks to Dr. Myron A. Leon for his continuous constructive critiques and everyday updates in literature.

My recognitions to the Department of Internal Medicine/Division of Endocrinology and to the Center for Molecular Medicine and Genetics for the constant help and support.

Thanks to my colleagues Dr. Biswajit Pati and Dr. Diane Deutsch for their support. I would like to recognize Mrs. Kassandra McGee, a summer student working at the Center for Molecular Medicine and Genetics on the summer of 1994, for her excellent assistance in the isolation of positive Ahsg clones.

Special thanks to Dr. Minoru Ko, at Center for Molecular Medicine and Genetics who kindly facilitated his laboratory for my research and helped me perform the mapping of mouse Ahsg gene.

I want to dedicate a very special thanks to my husband Tony, who spent long hours at the laboratory or at home, waiting for me to finish the experiments, and who was always there during the difficult times in graduate school. His love, optimism, care and everyday motivation helped me survive the long road. Tony has been part of my life for

twelve years and has dedicated his life to make me the happiest person. Tony, you have made this moment possible because of your infinite love and understanding, because of that, thanks for always being part of me.

Very special thanks to my father Modesto, to my mother Jesusa and brother Angel Luis, whom I left back at home to pursue this career, for their love and care in the distance and for their support.

I want to dedicate special recognition to the following institutions which have sponsored my graduate studies:

Minority Doctoral Program at Wayne State University

NIDDK, National Institutes of Health

Michigan Blue Cross Blue Care Foundation

Department of Biological Sciences

Center for Molecular Medicine and Genetics

Department of Endocrinology/ Internal Medicine, School of Medicine

Table of Contents

List of Tables

List of Figures

List of Abbreviations

kD	kilodalton
α_2-HSG	α_2-Heremans Schmid-glycoprotein
AHSG	human gene that encodes α_2-HSG
Ahsg	mouse gene that encodes α_2-HSG
bp	base pairs
IR	insulin receptor
TK	tyrosine kinase
TKA	tyrosine kinase activity
aa	amino acid
cpm	counts per minute
Milli Q water	filtered water
RT	room temperature
DMEM	Dulbecco's modified essential medium
BSA	bovine serum albumin
FBS	fetal bovine serum
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase-polymerase chain reaction
ORF	open reading frame
nt	nucleotide
rpm	revolutions per minute
pfu	plaque forming units

CHAPTER I

Introduction

Heremans and Schmid described α_2-HSG for the first time in 1960 (Heremans, 1960; Schmid and Bürgi, 1961), as a 49-60 kD human plasma glycoprotein secreted into the circulation by the liver. Schultze *et al.*, renamed the protein based on the electrophoretic mobility. Human α_2-HSG is a negative acute phase reactant protein (Lewis, 1983; Lebreton *et al.*,1979) and altered concentrations of α_2-HSG have been reported in several disease conditions including Paget's disease, osteogenesis imperfecta, lymphoma, leukemia and myelofibrosis (Ashton *et al.*,1980; Kalabay *et al.*,1991). Several functions have been attributed to α_2-HSG, including its involvement in immune response (Van Oss *et al.*, 1974), the chemotactic response of macrophages (Quelch *et al.*,1984; Malone *et al.*,1982), enhancement of phagocytic function of human monocytes (Lewis, *et al.*,1980; Lewis *et al.*,1981), bone mineralization (Dickson *et al.*, 1975; Schinke *et al.*,1996), bone accumulation (Triffitt *et al.*,1976; Mbuyi *et al.*,1982), calcification (Ashton *et al.*,1976; Keely *et al.*,1985) and as an inhibitor of insulin receptor tyrosine kinase activity (IR-TKA; Srinivas *et al.*, 1993, Fig. 1.1, Fig. 1.2). Recently, Jahnen-Dechent *et al.* (1997) demonstrated that mice deficient for α_2-HSG can develop ectopic microcalcifications in soft tissues, supporting the idea that α_2-HSG may operate as an inhibitor of apatite crystal growth *in vivo*.

Classified as a member of the cystatin superfamily (Dziegielewska *et al.*,1990), human α_2-HSG is synthesized as a type I secreted glycoprotein with a signal sequence of 18 amino acids (Lee *et al.*, 1987) and three major domains--two N-terminally located

cystatin domains, D1 and D2 (116-118 amino acids), and a single, proline-rich domain D3, (106-115 residues; Dziegielewska *et al.*,1990; Kellerman *et al.*,1989; Elzanowski, *et al.*, 1988; Fig. 1.3). Domain D3 harbors a proline rich region followed by a segment prone to proteolytic modification in human α_2-HSG. Many key amino acid residues and the position of cysteine residues are perfectly conserved between human α_2-HSG and the fetuins of bovine, sheep, pig, rat and mouse origin (Dziegielewska *et al.*,1987; Dziegielewska *et al.*,1990; Rauth *et al.*,1992; Lee *et al.*, 1987; Brown *et al.*,1992; Christie *et al.*,1987; Hayase *et al.*,1992), suggesting that the fetuins may be the non-human homologs of human α_2-HSG.

Rat fetuin, originally named pp63 (Le Cam *et al.*,1985), secreted by rat hepatocytes in the phosphorylated state (Le Cam *et al.*,1985), inhibits insulin-stimulated IR-TKA (Le Cam *et al.*,1989). The gene for rat fetuin maps to chromosome 11 and spans approximately 8 kb, containing seven exons separated by six introns of different sizes (Fig. 1.4). The rat fetuin cDNA sequence is similar to the cDNA sequences of both human α_2-HSG and bovine fetuin (Haasemann *et al.*, 1991).

When Yang *et al.* (1992) first reported the deduced amino acid sequence of the mouse fetuin cDNA, the question arose whether mouse fetuin was the true ortholog of human α_2-HSG. They suggested that the mouse protein take the name α_2-HSG instead of fetuin, because, unlike bovine fetuin, the mouse protein is not a major component synthesized by fetal liver (Yang *et al.*, 1992). Alignment of human and mouse α_2-HSG reveals a 60% amino acid identity between the two proteins, with the majority of the identical residues found in the N-terminal two-thirds of the protein. Three N-linked

glycosylation sites are present in mouse while only two are present in the human protein. Moreover, mouse α_2-HSG is 22 residues shorter than human α_2-HSG (Yang *et al.*, 1992; Fig. 1.5).

In this study, we report the mouse Ahsg genomic structure derived from sequencing and restriction mapping of exons 1, 2, 3 and 4 contained in a contiguous 4.3 kb segment of the gene. We have also sequenced a 154 bp region upstream from the transcriptional start site. The chromosomal location of mouse Ahsg has been mapped to the proximal region of chromosome 16 at 16 centimorgans, adjacent to the gene Diacylglycerol kinase 3 (Dagk3). Further, we demonstrated that recombinant mouse α_2-HSG inhibits insulin-stimulated IR autophosphorylation, IR-TKA and DNA synthesis, confirming that mouse α_2-HSG can play a role similar to the human homolog in modulating insulin action. Based on the structural features shared between the mouse and human genes, their syntenic chromosomal localization, and the IR-TK inhibitory activities shared between the two proteins, we suggest that the mouse Ahsg is not simply a family member, but the true ortholog of the human AHSG gene.

The Insulin Receptor

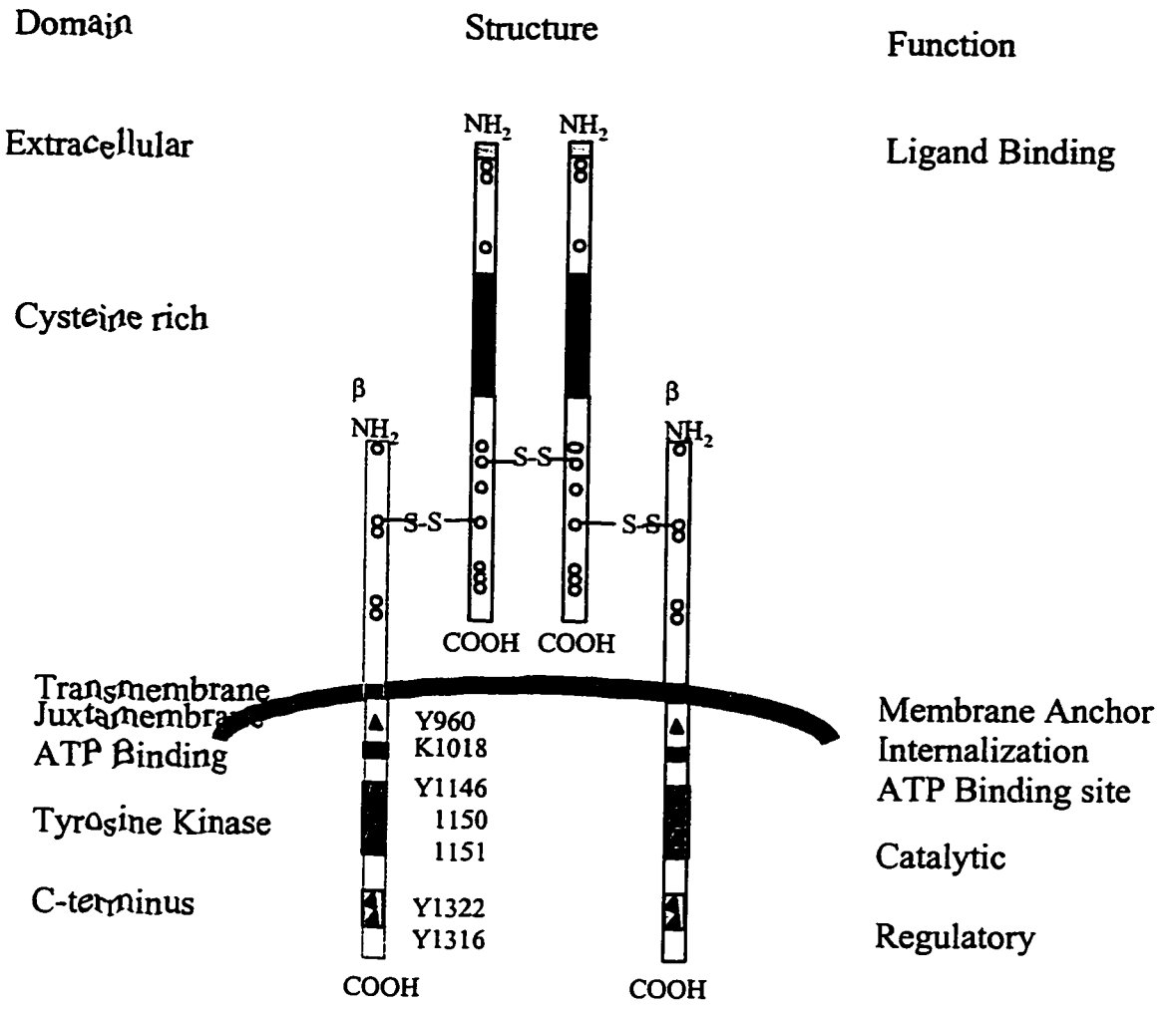


Fig. 1.1 The insulin Receptor. The insulin receptor is composed of several domains: the extracellular domain which serves as the ligand binding, the binding domain, the transmembrane domain involved in anchoring, the juxtamembrane domain involved in internalization, the ATP binding domain for ATP binding, the tyrosine kinase domain for catalytic activity and the C-terminus as the regulatory site. The circles in black represent the cystein residues and the triangles represent the tyrosine phosphorylation sites. (Olefsky *et al.*,1990; Van Obberghen *et al.*, 1994).

Fig. 1.2 Insulin signal transduction: effect of α_2-HSG. The star symbol represents the inhibition points of α_2-HSG. Srinivas *et al.*, 1995 demonstrated that human α_2-HSG inhibits insulin stimulated insulin receptor autophosphorylation *in vitro* and *in vivo* as well as exogenous substrate phosphorylation. α_2-HSG also inhibits tyrosine IRS-1 (insulin receptor substrate 1) phosphorylation and association with the p85 subunit of the phosphatidylinositol 3-kinase (PI3 kinase). Srinivas *et al.* also examined the downstream signaling pathway, demonstrating that α_2-HSG can inhibit insulin-induced association of Grb-2 (growth factor receptor-bound protein) and SOS (son of sevenless) association to IRS-1. The inhibition results in a decrease guanine nucleotide exchange in p21 Ras. α_2-HSG also can inhibit Raf phosphorylation in response to insulin as well as downstream mitogenic efect.

Insulin Signal Transduction: Effect of α_2-HSG★

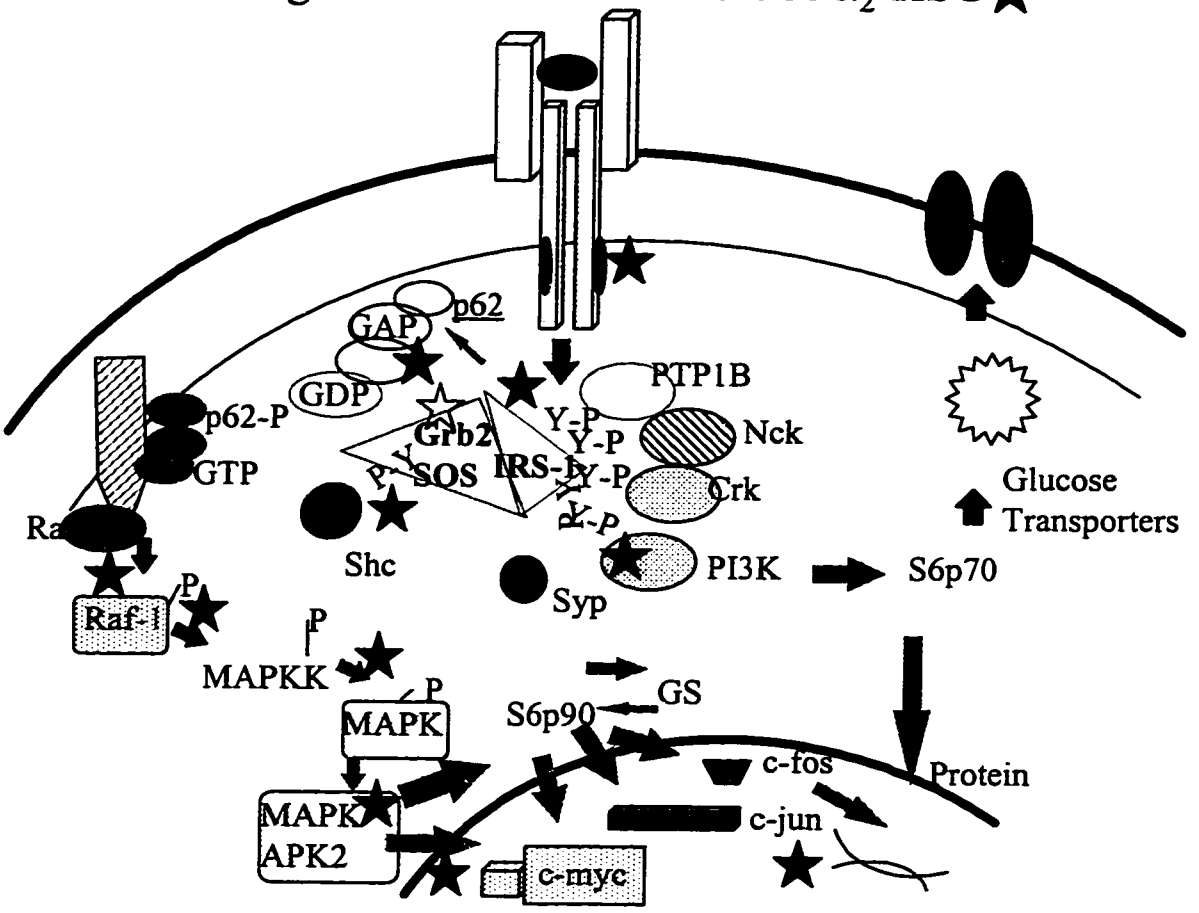
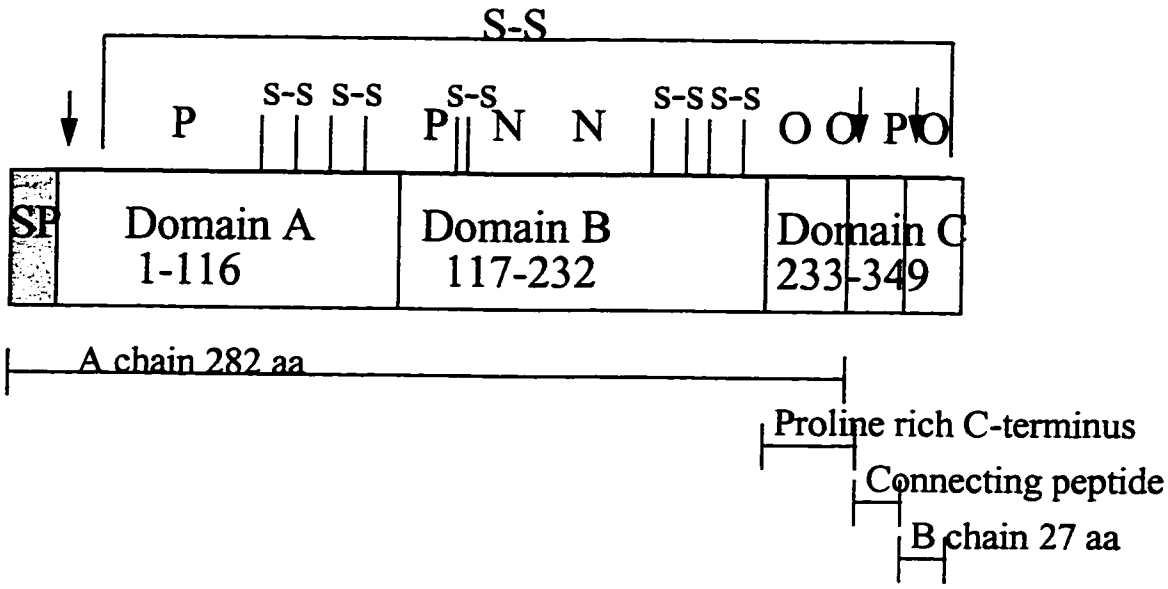
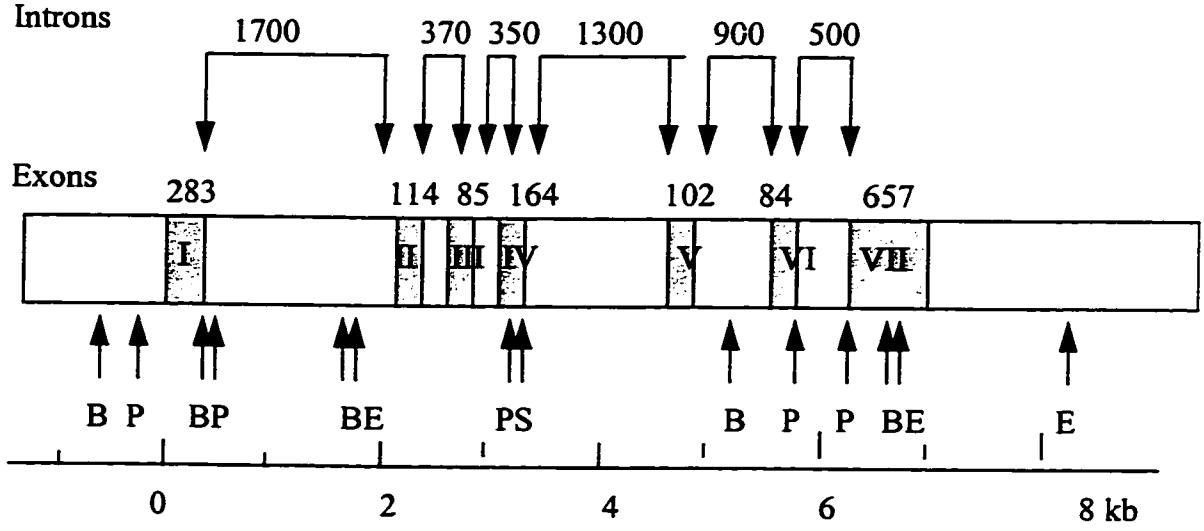


Fig 1.3 Schematic representation of α_2-HSG structure. α_2-HSG is composed of three domains: domain A, B and C. Domain A consist of residues 1-116 amino acid at the N-terminal, domain B is composed of 117-232 amino acids and domain C of 233-349 amino acid located at the C-terminus of the protein. Domains A and B are known as the cystatin domains which are similar in loop size, location and distance between the disulfide bonds. The protein also contains a signal peptide of 18 amino acids, the A chain of 282 amino a cids and the B chain of 27 amino acids. The two chains are separated by a connecting peptide. The A chain contains two potential serine phosphorylation sites (P), two N-glycosylation sites (N), two O-glycosylation sites and five intrachain disulfide bonds (SS). It also contains a proline rich region at the C-terminus. The B chain is composed of one O-glycosylation site. The connecting peptide region contains one potential serine phosphorylation site. The protein has several proteolytic cleavage sites (arrows): one after the signal peptide, two close to the 40 amino acid connecting peptide, and one tryptic proteolytic cleavage site (RK at 125-126; Auberger *et al.*,1989; Araki *et al.*,1989; Jahnen-Dechent *et al.*,1994).

Structure of α_2-HSG



Structure of Rat Fetuin (pp63) Gene



B: Bgl II; P: Pvu II; E: Eco RI; S: Sma I
Falquerho *et al.*, Gene 98, 209-216 (1991).

Fig. 1.4 Structure of rat fetuin:pp63 gene. Rat fetuin gene, originally named pp63 gene by Le Cam *et al.*,1985, maps to chromosome 11 and spans approximately 8 kb. It contains seven exons separated by six introns of different sizes.

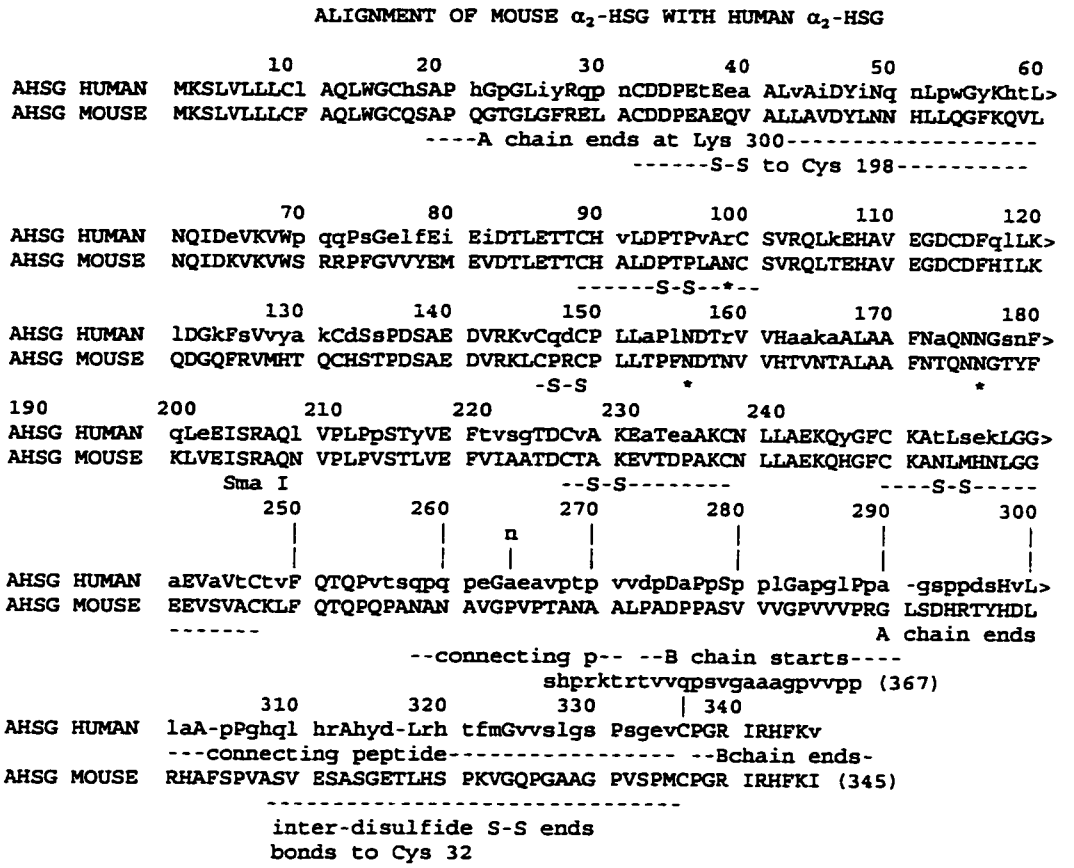


Fig. 1.5 Alignment of Human and Mouse α_2-HSG. This alignment reveals a 60% similarity in amino acids between the two proteins. The A and B chains are conserved in both proteins. The majority of the sequence similarity resides at the N-terminal two thirds of the proteins. Three N-linked glycosylation sites (*) are present in the mouse and only two are present in the human protein. The mouse α_2-HSG is 22 residues shorter than the human homolog. (Yang *et al.*, 1992).

CHAPTER II

Cloning of mouse Ahsg cDNA into a baculovirus expression system

Introduction

The proposed studies were designed to isolate and characterize the full mouse Ahsg cDNA and use it to express the mouse α_2-HSG protein in a baculovirus expression system. The initial experiments were performed to recover the full mouse Ahsg cDNA from liver by RT-PCR. The mouse Ahsg cDNA was sequenced and cloned into the plasmid pCRII. Further, the mouse Ahsg cDNA was cloned into the baculovirus vector to express the mouse α_2-HSG protein.

Autographa californica nuclear polyhedrosis virus (AcMNPV), member of the *Baculoviridae* family was used in this study to express the recombinant mouse α_2-HSG protein. In the course of infection two kinds of viral progeny are produced: extracellular viral particles and occluded viral particles. The occluded viral particles are composed of the polyhedrin protein (29kDa) which are important for the transmission of the virus. These occlusions also serve as protectants from environmental factors that may lead to inactivation. The viral cycle starts by larvae ingesting the virus and the polyhedra at the contaminated plants (Fig 2.1). The alkaline insect gut releases the virus which then replicates in the cells. Later on, it spreads to different tissues by budding mechanisms. The polyhedrin gene is not necessary for infection or replication, making the system efficient in replacing this gene for the gene of interest. In our study, mouse Ahsg was under the strong polyhedrin promoter and direct expression of the gene was positively identified by the result of occlusion negative particles.

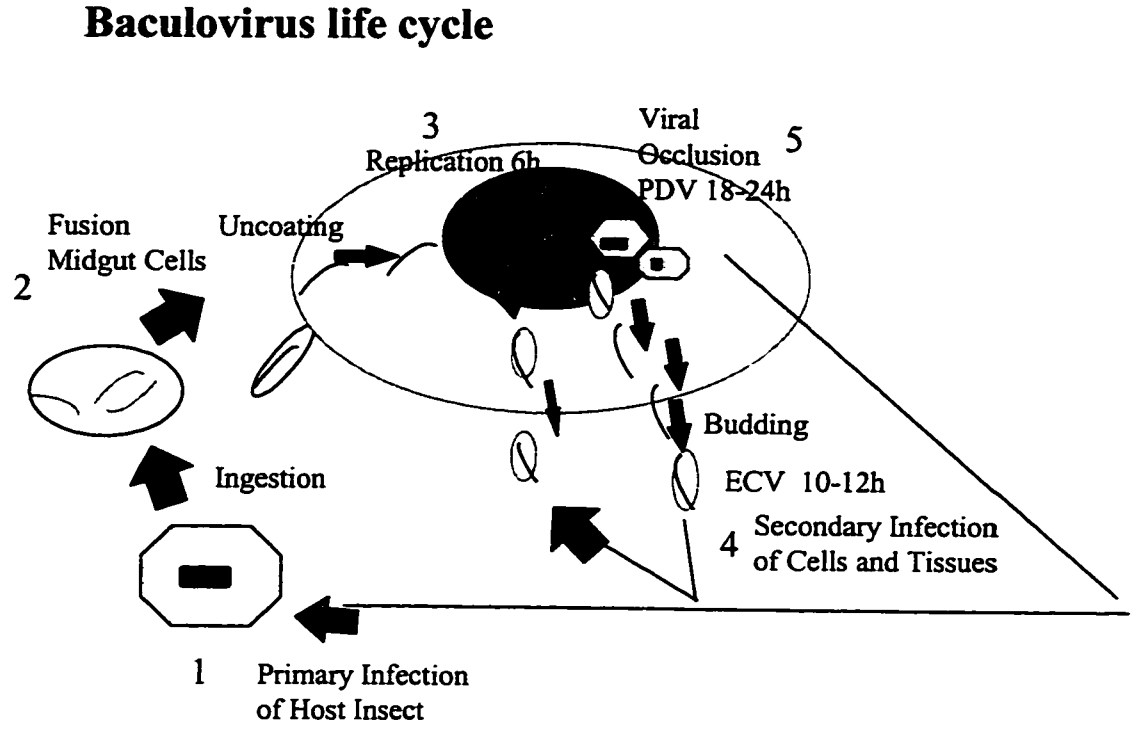


Fig. 2.1 Baculovirus life cycle. The cycle starts when the larvae ingest the virus. The second step represents the invasion of the insect gut cells by the virus. The virus then starts to uncoat and replication develops for 6 hours. At this step the virus can be released at 10-12 hours by budding and a secondary infection occurs invading other cells or tissues nearby; or the viral occlusion particles can be packed after 18-24 hours ready to infect another host cells.

The polyhedrin gene was contained in the wild type viral DNA (AcMNPV). The wild type virus was linearized in order to allow recombination to occur with the BlueBacIII baculovirus vector. The wild type polyhedrin gene was replaced by homologous recombination for the mouse Ahsg ORF in pBluebacIII. Sf-9 (*Spodoptera frugiperda*) cell line was co-transfected in presence of the wild type viral DNA and the transfer vector (Fig.2.2). The occlusion negative viral particles were selected as recombinants determined by the blue color (lacZ expression).

Material and Methods

Recovery of the full length mouse Ahsg cDNA

Initial studies were conducted to isolate and identify the mouse homolog of human AHSG. The first approach was to recover the full mouse Ahsg cDNA synthesized from mouse liver cDNA. A mouse cDNA encoding 1095 bp of open reading frame was cloned from liver poly(A+) RNA using reverse transcriptase (RT)-polymerase chain reaction (PCR). Two primers were used to amplify this fragment, VIV1 and VIV2:

VIV1 5'-CTGCCAATCCGCTCCACAAGGTA-3'OH

VIV2 5'-TGTGGTATTGCTTTGTCAGTGGA-3'OH

These primers were synthesized based on the cDNA sequence published by Funmei Yang *et al.*,1992, accession number S96534. The RT/PCR fragment was cloned into the pCRII plasmid, host XL-1 blue MRF', (Invitrogen, Carlsbald, CA) and selected white colonies on TKXI plates (tetracycline, kanamycine, X-Gal, and IPTG). The positive clone was named pTA.mAHSG#3 and was verified using restriction enzymes and by sequencing.

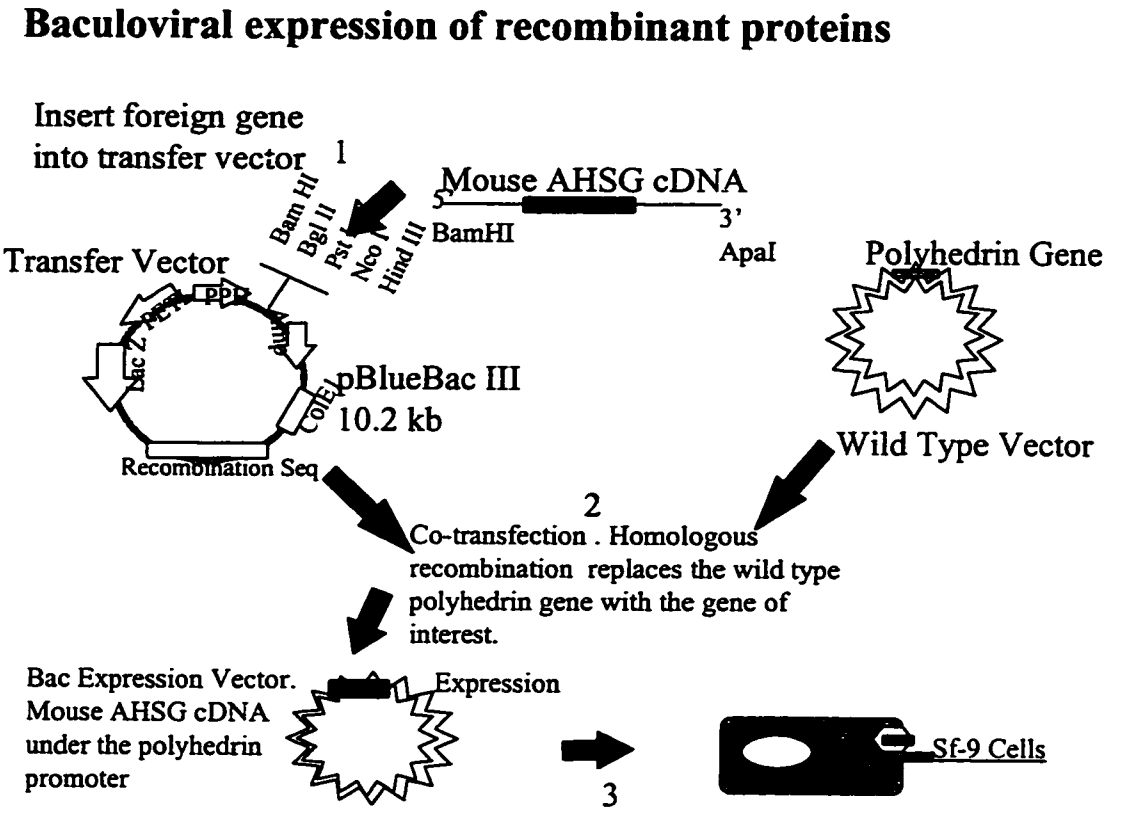


Fig. 2.2 Baculovirus expression of recombiant protein. Mouse Ahsg was cloned into a BamHI-ApaI site in the baculovirus vector pBlueBacIII (transfer vector). This vector was co-transfected in presence of a wild type baculoviral vector containing the polyhedrin gene. The polyhedrin gene was replaced by the wild type polyhedrin gene by the mouse Ahsg cDNA. The mouse Ahsg cDNA was under the expression of a strong polyhedrin promoter. Mouse α_2-HSG protein was expressed in Sf-9 insect cells and purified using jacalin affinity column.

It was observed that the mouse cDNA clone produced (pTA.mAHSG#3a) lacked an ATG start codon. The cDNA insert in this clone contained most of the open reading frame (ORF), plus 101 bp 3-UT, but lacked 44 nucleotides at the 5' end of the open reading frame. Two primers were engineered, (SSF1 +32 to +52 of exon 1 and SSB3 a 22-mer primer in exon 4) to recover the full length mouse cDNA.

SSF1 5'-GGATCCTGACATTTGCCCATTTTCC-3'OH

SSB3 5'-CGGTGTGGACCACGTTGGTATC-3'OH

The SSF1 primer was designed to have an artificial BamHI (GGATCCTGACAATTTGCCCATTTTCC) site to facilitate cloning. Using mouse poly (A+) RNA (Clontech, Palo Alto, CA) in RT/PCR with primers, SSFI and SSB3, an amplified product of 535 bp was obtained. The amplified product contained the expected 45 bp upstream from the start codon and 490 bp of the mouse Ahsg ORF. This product was cloned into the TA cloning system plasmid pCRII (Invitrogen) as pTA.mAHSG5 and transformed into the XL-1 Blue *E. coli* strain. Equimolar concentrations of plasmid pTA.mAHSG#3A (the 3' end of the mouse cDNA encoding +118 to +1212 bp) and plasmid pTA.mAHSG5 (the 5' end of the mAHSG encoding +31 to 563 bp) and the primers SSF1 and VIV2 were used to create a PCR product of the full length mouse AHSG cDNA by gene short ovelapping extension (Fig.2.3). The experiment was engineered to obtain an overlap of 446 bp, facilitating the recovery of the full-length mouse AHSG cDNA (1182 bp, +31 to +1212). The full length PCR mAHSG product was ligated into the pCRII plasmid. The insert was designed to have a "portable" BamHI-EcoR1 1.2 kb fragment containing the entire mouse Ahsg ORF.

Directional cloning was performed using 100 ng of the vector plasmid and 200 ng

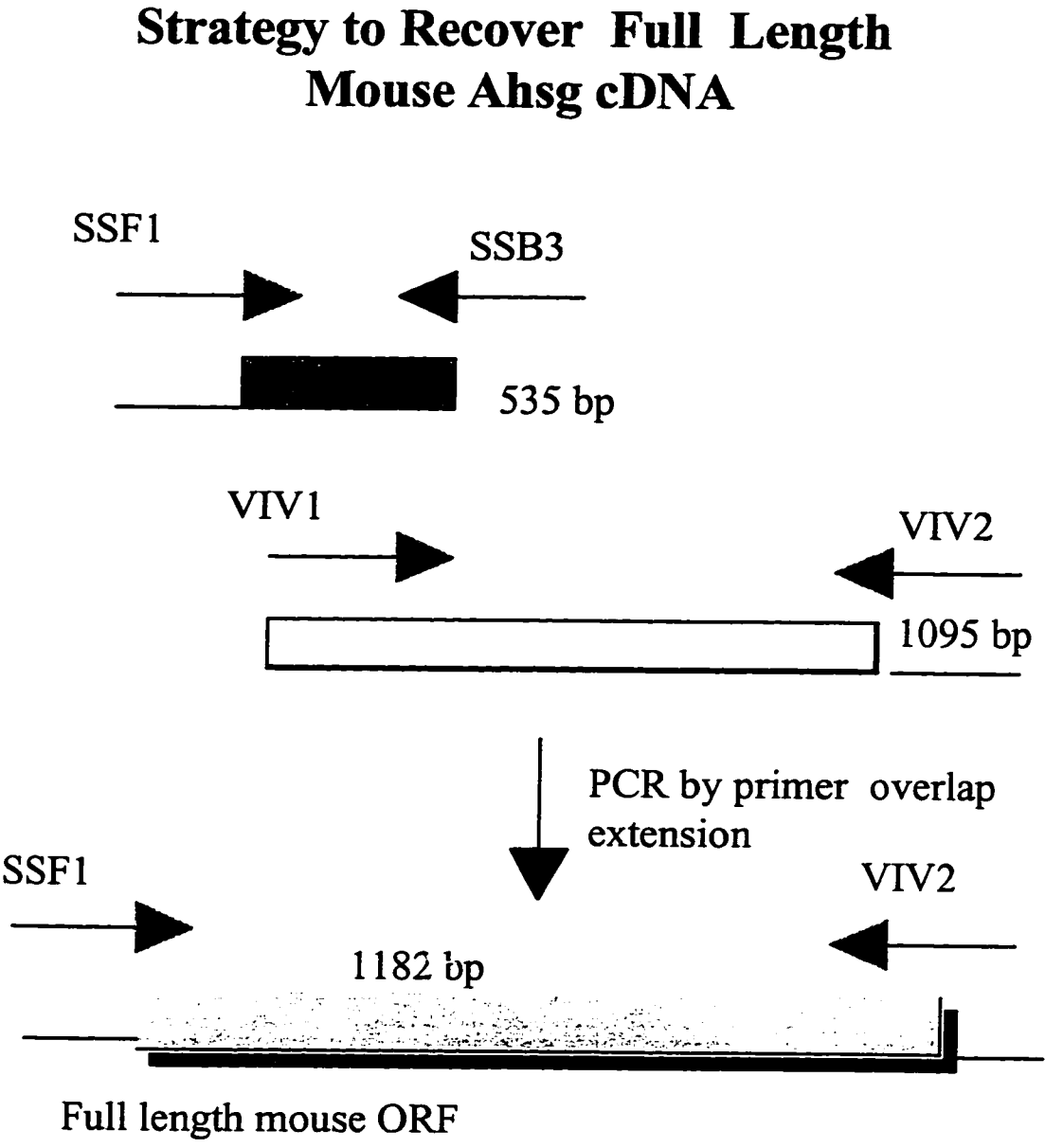


Fig. 2.3 Strategy to recover the full mouse Ahsg cDNA. Two primers were engineered (SSF1 and VIV2) to create a PCR product of the full length mouse Ahsg cDNA. The experiment was engineered to obtain an overlap of 446 bp, facilitating the recovery of the full-length mouse AHSG cDNA (1182 bp, +31 to +1212). The full length PCR mAHSG product was ligated into the pCRII plasmid. The insert was designed to have a "portable" BamHI-EcoR1 fragment containing the entire mouse AHSG ORF.

of the insert in presence of 1 μl of each T4 DNA ligase and 10x ligase buffer in a volume of 10 μl. This reaction was incubated overnight at 12°C. The pCRII plasmid was self-ligated in the absence of the insert as a parallel ligation control allowing to determine the frequency of self-ligation (blue colonies) and to determine the frequency of spontaneous white colonies due to deletions in the vector generated during digestion with EcoR1 and BamHI. One microliter of the plasmid-ligation reaction was mixed with 30 μl of *E. coli* XL-1 blue competent cells (Stratagene, San Diego, CA). The transformations were performed according to the manufacturer's directions. A total of 100 μl of XL-1 blue competent cells were mixed with 1.4 μl β-Mercaptoethanol (14.5 mM) and incubated on ice for 10 min. A volume of 5 μl of the ligation reaction was added to the treated cells and incubated for 30 min on ice. The cell-ligation mixture was incubated for 45 sec in a 45 °C water bath and placed on ice for 2 min. One hundred microliters of the transformation mixture were plated onto TAXI (tetracycline, ampicillin, X-Gal and IPTG) plates containing 12.5 μg/ml tetracycline, 100 μg/ml ampicillin, 40 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase), 1 mM isopropylthio-β-galactopyranoside (IPTG). The plates were incubated overnight at 37°C. Single white colonies representing transformed *E. coli* containing the mouse Ahsg cDNA insert were transferred into 50 μl of TE buffer (TE; 10 mM Tris-HCL, pH 7.8, 1mM EDTA-NA_3) and boiled for 5 minutes to extract the inserts. One microliter of the boiled TE suspension was used as a template in a 20 μl PCR reaction using VIV1 and VIV2 primers. Successful recombinants generated a 1.23 kb band detectable on an ethidium bromide stained 1% agarose gel. The mouse Ahsg ORF plasmid was isolated from a 3 ml overnight culture grown in Luria-

Bertani (LB) broth containing 100 μg/ml ampicillin. The insert orientation within the plasmids was verified by restriction mapping using PvuII enzyme (New England BioLabs, Beverly, MA). The resulting map was compared with the expected map of the human cDNA AHSG. The plasmid was sequenced from a combination of dye-primer and dye-terminator automated sequencing reactions.

Cloning of the mouse Ahsg cDNA into the baculovirus transfer vector

The baculovirus transfer vector pBlueBacIII (10242 bp, Invitrogen, Carlsbad, CA) was digested with NcoI and blunt ended using Klenow fragment of *E. coli* DNA polymerase I (Pharmacia, Piscataway, NJ) simultaneously. An excess of non phosphorylated ApaI linker was added to this reaction to create a new ApaI site in the transfer vector. This vector was sequenced using the baculovirus polyhedrin primers: forward (+794) 5' TTTACTGTTTTCGTAACAGTTTTG-3' and reverse (-44) 5'-CAACAACGGACAGAATCTAG-3'. The plasmid was recircularized using a ligation reaction. The product was incubated with NcoI to destroy parental pBlueBacIII DNA. The plasmid pBlueBacIII/ApaI was linearized with BamHI and ApaI and the 1.23 kb mouse AHSG cDNA insert was ligated to pBlueBacIII/ApaI at the BamHI-ApaI site, at a ratio of 1/25 insert:plasmid, resulting in pMUSBacα3 (11,440 bp). The plasmid was analyzed with restriction enzyme PvuII and sequenced using primers SSF1 and VIV2. Super competent *E. coli* cells, XL-1 blue, tetracycline resistant, were transformed with pMUSBacα3 and selected. Minicultures were prepared and purified using Quiagen kits (Chatsworth, CA).

Expression of the mouse α2-HSG protein in the baculovirus

Sf-9 cells (Gibco, Gaithersburg, MD) were seeded (3 x 10^4) onto 60 mm tissue culture plates using Grace's Insect Supplemented Medium (Gibco). A total of 5 μg of linearized wild-type baculovirus DNA was mixed with 2 μg of recombinant plasmid DNA containing the mouse Ahsg construct (pMusBacα3) and infection was carried out using the BaculoGold system (PharMingen, San Diego, CA). A mock infection without the virus was performed, to serve as a negative control. Two positive controls were used, one with the wild type virus (*Autographa californica nuclear polyhedrosis virus*, AcMNPV, member of the *Baculoviridae* family containing the polyhedrin gene) and another with 2 μl of the human AHSG plasmid construct (pBlueBacα2, Srinivas *et al.*, 1995). The reactions were incubated for 5 min at room temperature. A total of 1 ml of BaculoGold buffer B was added to each of the reactions and mixed. Immediately, 1 ml of the BaculoGold transfection buffer B/MIX was added to the plated cells dropwise until a precipitate was formed. The transfections were incubated in a closed container at 27°C for 5 days, after which 150 μl of the infected supernatant were removed from each of the plates and added to already seeded cells (3x10^6 cells/60 mm plates) for further purification. Several dilutions (10^{-3}, 10^{-4}, 10^{-5}) of transfected virus were prepared and 300 μl of each dilution were mixed with 2.7 ml of Grace's supplemented medium. The mixture was added to seeded cells and incubated for 1 hour at room temperature. X-Gal agarose was prepared to a final concentration of 2%. The overlay agarose was prepared in 2x Grace's medium supplemented with 20% FCS and 40 μg/ml gentamicin. After a 1 hour incubation, the medium was removed, the agarose diluted to 1% and gently poured over the plates. The plates were kept in a closed container with a wet towel to provide

humidity until blue plaques developed, approximately 5 days. These plaques were purified as single clones. A viral DNA preparation was required to verify using PCR the presence of the mouse Ahsg ORF. A sample conatining 500 µl of recombinant virus was recovered and centrifuged at 5000 rpm for 3 min. The supernatant was transferred to a fresh tube and 500 µl of 20% polyethylene glycol (PEG) in 1.0 M NaCl solution were added. The tube was inverted twice to mix and allowed to stand at room temperature for 30 min. The tube was centrifuged at 12000 rpm for 10 min at room temperature. A total of 100 µl of sterile water were added to the pellet followed by 10 µl of Proteinase K (5-10 mg/ml) and allowed to incubate at 50°C for 1 hour. Equal volume of phenol:chloroform (1:1) was used to extract the DNA. The tube was centrifuged for 5 min and the upper aqueous phase was transferred to a fresh sterile tube. The DNA was precipitated by adding 1/10 volume 3M NaOAc, 5 µl of glycogen, and 2 volumes of 100% ethanol then incubated at -20°C for at least 20 min. The tube was centrifuged at 14000 rpm for 15 min at 4°C. The pellet was washed with 80% ethanol and spun at 14000 rpm for 5 min to remove the traces of ethanol. The pellet was resuspended in 10 µl of sterile water. Positive recombinant extracted DNAs were identified using PCR (baculoviral primers +794 and -44) and re-amplified on Sf-9 cells to yield a high titer of pure mouse α_2-HSG recombinant baculoviral stock. Expanded recombinant baculoviral stocks were used to transfect cabbage looper HighFive™ cells (Invitrogen, Carlsbad, CA).

Purification of mouse α_2-HSG

Supernatants collected after 72 hours were purified using affinity chromatography

on jacalin lectin columns (used for affinity chromatography of glycoproteins), (Sigma, St. Louis, MO), washed with 100 mM Tris-pH 7.4 and eluted with 25 μM melibiose (Sigma). Proteins (50 μg/ml) separated on 12% SDS-PAGE were electroblotted to nitrocellulose, blocked with 8% non-fat dry milk and probed with a polyclonal rabbit antibody specific for rat fetuin (kind gift from Dr. Le Cam).

Results

A full-length cDNA encoding the entire 1035 nt open reading frame (ORF) was cloned from liver poly(A+) RNA using reverse transcriptase (RT)-polymerase chain reaction (PCR). The cDNA was amplified in two segments--a 535 bp N-terminal segment (using primers SSF1, 5'-GGATCCTGACATTTGCCCATTTTCC-3'OH, and SSB3, 5'-CGGTGTGGACCACGTTGGTATC-3'OH) and a 1095 bp C-terminal segment (Fig. 2.4, Fig.2.5) (using primers VIV1, 5'-CTGCCAATCCGCTCCACAAGGTA-3'OH and VIV2, 5'-TGTGGTATTGCTTTGTCAGTGGA-3'OH). A continuous cDNA of 1182 bp was then amplified from the two segments using PCR and short overlap extension (primers SSF1 + VIV2; SSFI was designed to have an artificial BamHI site) and the PCR product cloned into a BamHI-EcoRI sites in the plasmid pCRII (Invitrogen, Carlsbad, CA) (Fig. 2.5; Fig. 2.6; Fig. 2.7).

A baculoviral transfer vector, pMusBaca3 (11,440 bp; Fig.2.8) was created using a 1.2 kb BamHI-ApaI segment of the mouse Ahsg cDNA featuring an intact 1035 bp open reading frame (Fig.2.9), as described in Materials and Methods. This transfer vector (2 μg) was transfected into Sf 9 cells along with 5 μg of the wild-type baculoviral DNA, and blue plaques selected from 2% agarose plugs stained in X-Gal. Twice-purified viral plaques were expanded at 27°C, and recombinant viral stocks checked for homogeneity

using PCR (baculoviral primers -44, 5'-TTTACTGTTTTCGTAACAGTTTTG-3'OH; and +794, 5'-CAACAACGCACAGAATCTAGC-3'OH; Fig.2.10; Table 2.1; Table 2.2) Expanded recombinant baculoviral stocks were used to transfect cabbage looper HighFive™ cells, and supernatants collected after 48-72 hours. Supernatants were purified using affinity chromatography on jacalin lectin columns and proteins eluted with 25 μM melibiose were quantitated, separated on 12% SDS-PAGE and electroblotted to nitrocellulose. Probing of this membrane with a polyclonal rabbit antibody specific for rat fetuin revealed two prominent bands of 60 and 66 kD (Fig. 2.11) in addition to a fainter band of a slightly smaller apparent molecular weight.

Discussion

The full mouse Ahsg cDNA was generated by RT-PCR as a 1.24 kb fragment. The sequence of mouse Ahsg cDNA revealed high similarity with the rat fetuin cDNA. The mouse Ahgs cDNA was cloned as a BamHI-ApaI fragment in the baculovirus transfer vector pBlueBAcIII. Expression of mouse α_2-HSG protein was achieved and a high titer of recombinant baculoviral stock was recovered. Positive plaques were identified and re-amplified in Sf-9 and Hi-5 cells to yield a pure titer of mouse α_2-HSG protein. The crude recombinant protein was purified using jacalin chromatography column. The eluates were loaded in a 12% SDS-PAGE gel and inmmunoblotted against rat fetuin: pp63. This western blot revealed two prominent bands at 60 and 66 kD. These bands correlated with the molecular weight of the human α_2-HSG protein. These results indicate that expressed mouse α_2-HSG is the homolog of human α_2-HSG.

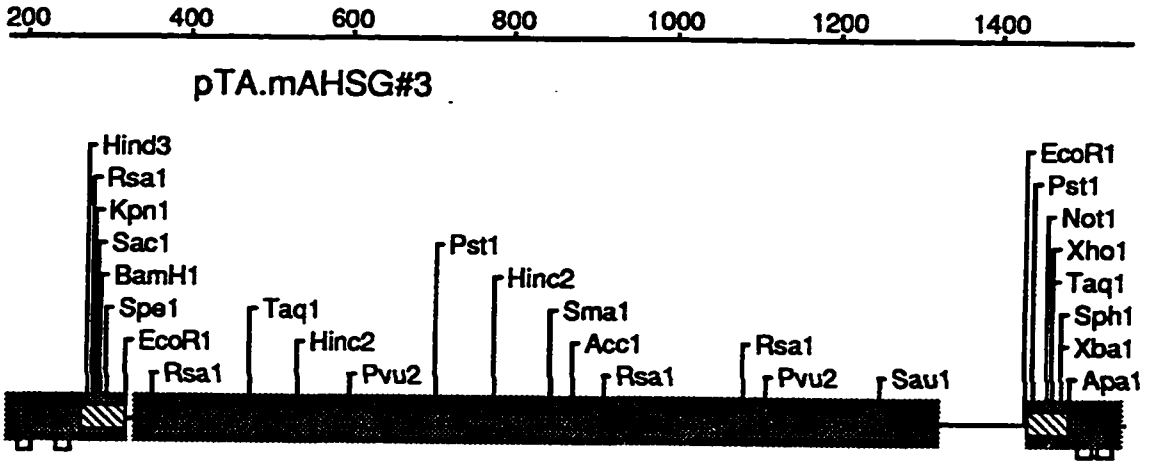


Fig. 2.4 1095 bp open reading frame: clone pTA.mAHSG#3. This mouse Ahsg ORF fragment was recovered using RT-PCR form mouse liver. Two primers were engineered to amplify the segment: VIV1 and VIV2 based on the cDNA sequence published by Yang *et al.*, 1992. The fragment was clone into the pCRII vector and analyzed by restriction mapping. This analysis demonstrated that the cDNA clone lacked the ATG start codon. The insert contained most of the mouse Ahsg ORF, a 101 bp of the 3' untranslated region, but lacked of 44 nucleotides at the 5' end of the open reading frame.

```
   1 CTGCCAATCC GCTCCACAAG GTACAGGACT GGGTTTTAGA GAATTGGCTT GTGATGATCC
  61 AGAAGCAGAG CAAGTAGCTT TGTTGGCCGT GGACTACCTC AATAATCATC TTCTTCAGGG
 121 ATTCAAACAG GTCTTGAATC AGATCGACAA AGTCAAGGTG TGGTCTCGGC GGCCCTTCGG
 181 AGTGGTGTAT GAGATGGAAG TTGACACACT GGAGACCACT TGCCATGCTT TGGACCCCAC
 241 CCCGCTGGCA AACTGTTCTG TGAGGCAGCT GACTGAGCAC GCGGTGGAGG GAGACTGTGA
 301 CTTCCACATC CTGAAACAAG ACGGCCAGTT CAGGGTGATG CACACCCAGT GTCATTCCAC
 361 CCCAGACTCT GCAGAGGACG TTCGTAAGTT GTGCCCACGG TGCCCACTCC TGACTCCGTT
 421 CAACGATACC AACGTGGTCC ACACCGTCAA CACTGCCCTG GCTGCCTTCA ACACACAGAA
 481 TAATGGAACC TATTTTAAAC TGGTGGAGAT TTCCCGGGCT CAAAATGTGC CTCTCCCAGT
 541 GTCTACTCTG GTGGAGTTTG TAATAGCTGC CACTGACTGT ACTGCAAAAG AAGTCACAGA
 601 TCCAGCCAAA TGCAACCTGC TGGCAGAGAA GCAACATGGC TTCTGCAAGG CAAATCTCAT
 661 GCATAATCTT GGTGGGGAAG AAGTTTCAGT GGCCTGCAAG TTATTCCAAA CACAGCCCCA
 721 GCCAGCCAAT GCCAACGCAG TAGGTCCCGT ACCCACAGCG AATGCAGCCC TACCAGCTGA
 781 CCCACCTGCA TCTGTGGTGG TGGGACCTGT GGTGGTTCCA CGAGGACTTT CAGACCACCG
 841 AACTTACCAC GACCTACGCC ACGCCTTCTC TCCTGTGGCC TCGGTGGAGT CGGCCTCGGG
 901 AGAAACTCTT CATTCTCCTA AGGTGGGCCA GCCTGGTGCT GCTGGTCCAG TGTCCCCCAT
 961 GTGCCCAGGG AGGATCAGAC ACTTCAAAAT CTAGGCTTGA TTCGGGGAAG TAAGGTTTTG
1021 GCAGACAGGA CATAGCCACC ACTGAAGCTG GGGGCGGGGA GGGGGTGGCT TGTCCACTGA
1081 CAAAGCAATA CCACA
```

Fig. 2.5 Sequence of a 10 95 bp ORF fragment. This figure represents the segment of mouse Ahsg ORF obtained by RT-PCR using mouse liver. The fragment contained most of the mouse Ahsg cDNA and was cloned in plasmid pCRII vector.

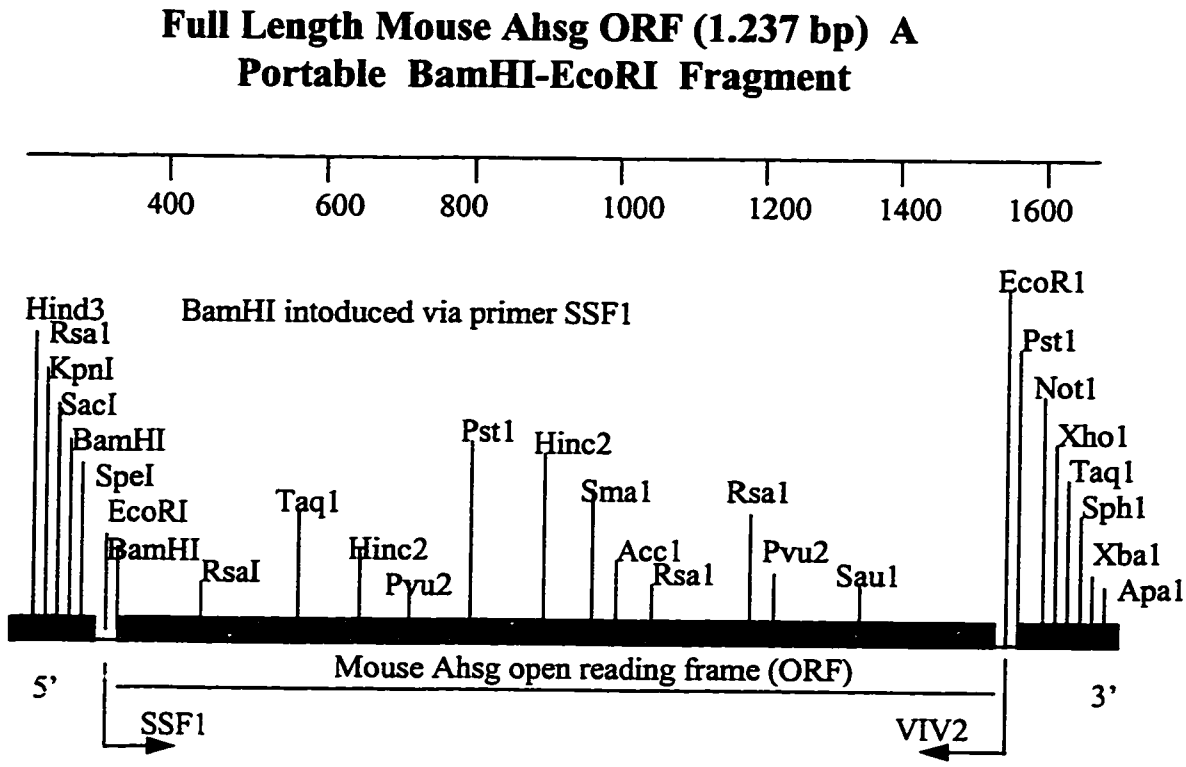


Fig. 2.6 Full mouse Ahsg ORF. The full Ahsg cDNA was recovered by gene overlapping extension PCR using two fragments of cDNA. The full mouse Ahsg cDNA was ligated into pCRII plasmid. The insert was designed to have a portable BamHI-EcoRI 1.2 kb fragment containing the entire mouse Ahsg ORF.

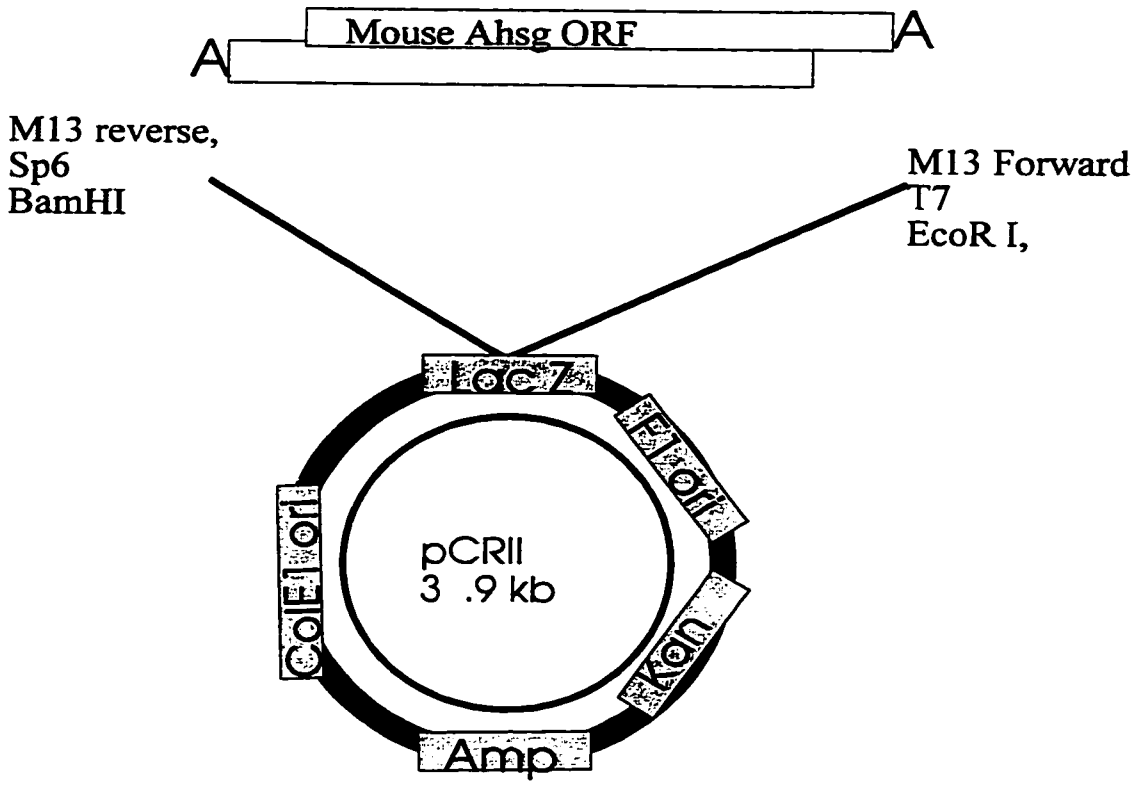


Fig. 2.7. Cloning of the full mouse Ahsg ORF into the pCRII vector. The full mouse Ahsg cDNA was cloned into the BamHI-EcoRI sites in the pCRII plasmid. This plasmid contains ampicillin and kanamycin selection markers and Lac Z operon for color selection of the positive colonies.

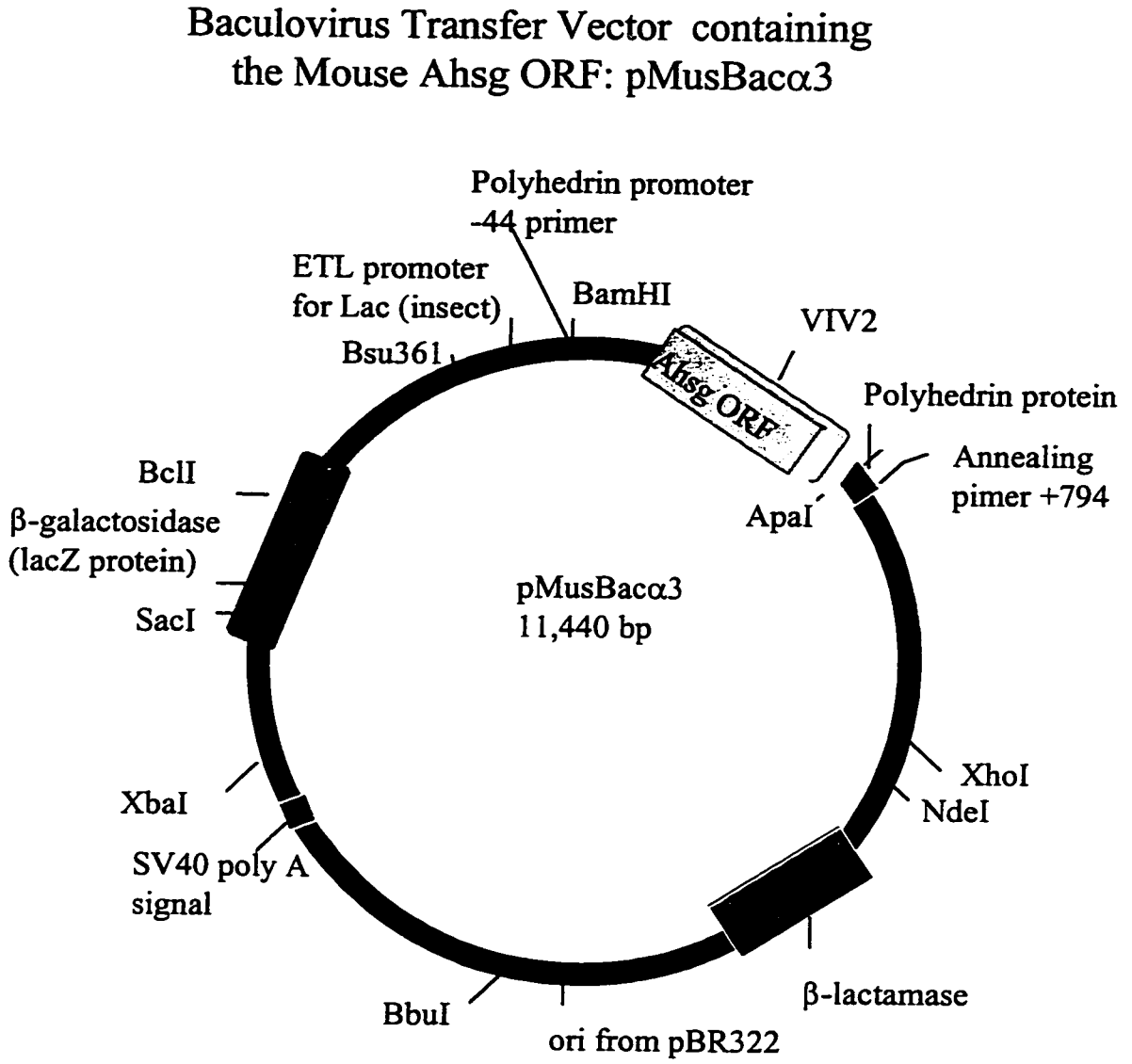


Fig. 2.8 Baculovirus transfer vector containing the mouse Ahsg ORF: pMusBacα3. The complete mouse Ahsg cDNA was cloned into the baculovirus vector pBlueBacIII resulting in vector pMusBacα3. This vector was co-transfected in presence of the wild type baculovirus vector. The wild type polyhedrin gene was replaced for the mouse Ahsg cDNA by homologous recombination. The expression of mouse protein α_2-HSG was driven by the strong polyhedrin promoter. Positive plaques were selected by Lac Z marker and Sf-9 insect cells were infected with the recombinant protein for amplification of the virus titer.

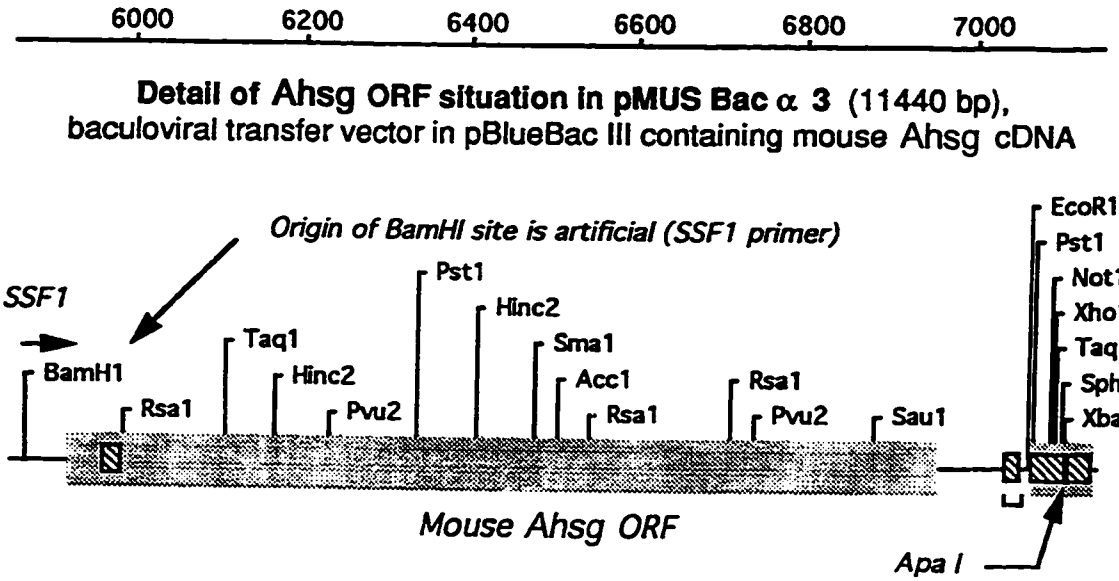


Fig. 2.9 Ahsg ORF:pMucBacα3. This figure represents the restriction map of the full mouse Ahsg cDNA cloned in the baculovirus vector pBlueBacIII at BamHI-ApaI sites.

Table 2.1 PCR conditions to analyze the presence of the full mouse Ahsg ORF in pMusBacα3

Reagents	Volumes
25 mM $MgCl_2$	2.5 µl
10 x Taq Buffer	2.5 µl
2 mM dNTP's	2.5 µl
10 pM Primer PF+794	1.0 µl
10 pM Primer PR -44	1.0 µl
Taq Pol 5U/ µl	0.13 µl
water	10.37 µl

Table 2.2 PCR conditions to analyze the full mouse ORF.

Step	Time	Temp	Cycles
Hot start	5 min	94 °C	1x
Denaturation	30 sec	94 °C	31x
Annealing	1 min	58 °C	
Extension	2 min	72 °C	
Extension	10 min	72 °C	1x
Soak		4 °C	

PCR Analysis of pMUSBacα3

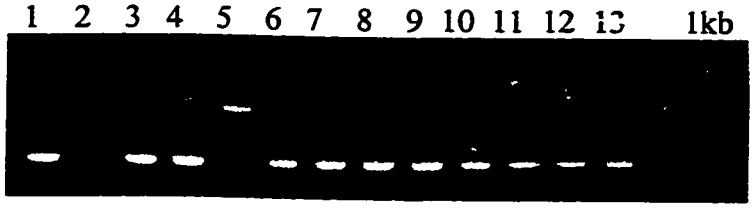


Fig. 2.10 PCR analysis of pMusBacα3. The 1.2 kb full mouse Ahsg ORF was amplified from pMusBacα3 using baculoviral primers +794 and -44. The two bright bands correspond to 1.2 kb fragment identified in positive clone 2 and 5.

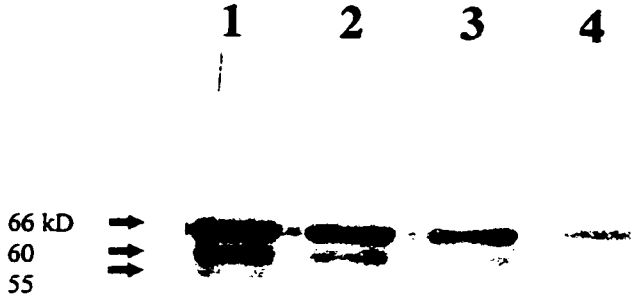


Fig. 2.11 Recombinant mouse α_2-HS-glycoprotein expressed in a baculoviral system. Immunoblot of mouse α_2-HSG sythesized in insect cells, partially purified by lectin chromatography. Numbers above indicate fractions eluted from the jacalin affinity column. Two forms are prominent, with molecular weights of 60 and 66 kD.

CHAPTER III

Screening genomic clones harboring the mouse Ahsg gene

Introduction

These studies were performed to isolate the mouse Ahsg gene. The gene sequence, first discovered in this study, revealed a sequence similarity to the homolog of the human AHSG and the rat fetuin gene. The mouse Ahsg contains several interesting binding sites for transcription factors and regulatory sites probably involved in the signaling of the gene and regulation of the translation of the mouse α_2-HSG protein.

An Svj 129 library, constructed in λDASH2 (Stratagene, LaJolla, CA) using spleen genomic DNA from male Svj mice, was screened for the mouse Ahsg gene. After infection of PLK-17 cells, plaques were generated at 50,000 plaques per plate, and lifted onto 137 mm Ø Nytran filters (0.45 μ, Schleicher and Schüll, Keene, NH). Filters were hybridized at high stringency (50% formamide, 43°C) with a [^{32} P]-labeled cDNA probe (1.2 kb) representing the mouse Ahsg cDNA.

Materials and Methods

Mouse genomic library in λDASH II

An Svj 129 library, constructed with spleen genomic DNA from male Svj mice, was used to screen for the mouse Ahsg gene. The library was contained in a λDash II (17-21 kb) vector (Stratagene, LaJolla, CA)(Fig. 3.1), a kind gift of Dr. Roger Askew (University of Cincinnati). In this library, the insert was prepared by pooling Sau 3A

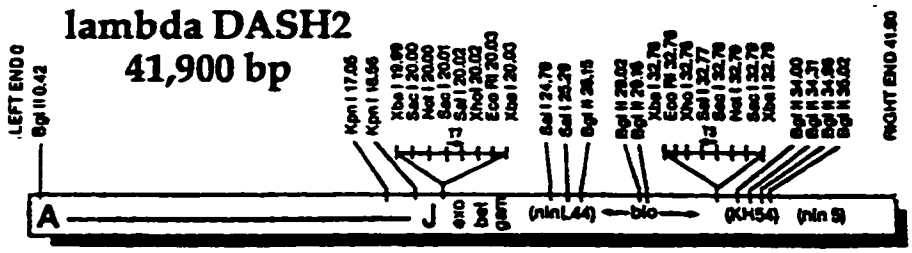


Fig. 3.1 Lambda DASH II vector. The mouse genomic library used to screen the mouse Ahsg gene was contained in the lambda DASH II vector (Stratagene,La Jolla, CA). The vector is 41,900 bp. The genomic library inserts contained in the library are from 17-21 kb and can be analyzed by PCR or sequencing using T3 and T7 promoters. The fragments were ligated to BamHI ends of the phage. The phage can be mapped by indirect end labeling of partial digest-southern blots with oligos of the T3 and T7 promoter sequences. The genomic library was plated using PLK-17 *E.coli* strain and hybridized with mouse Ahsg cDNA under high stringency. Positive clones were identified in an autoradiography and purified. The mouse Ahsg gene was identified by restriction digests using BglII and EcoRI as an 8kb gene.

partial digests and size selecting 17-21 kb fragments. These fragments were ligated to BamHI ends of λDASH2. The phage can be mapped by indirect end labeling of partial digest-southern blots with oligos of the T3 and T7 promoter sequences. First, the insert should be released by complete digestion with Xba I, Sal I or Not I, located outside of the T3 and T7 promoters. A series of decreasing partial digests by an enzyme of interest is run on a gel and blotted. Hybridization of this Southern blot with one of the promoter oligos yields a band sizes which represents distances of restriction sites from the end of the insert.

The genomic library was plated together with the infected PLK-17 bacterial cell line (*hsdR-M-, mrcA-,mrcB-,sup E44, lacZ,galK2,galT22,met B1,hsdR2*) at approximately 50,000 plaques per plate.

Preparation of the plating bacteria

Host *E. coli* strain PLK-17 was used for plating the genomic library. A culture tube containing 125 ml of LB medium supplemented with 10 mM $MgSO_4$ was inoculated with a single colony of PLK-17. After overnight incubation, the culture was diluted by 10 fold using warm LB/$MgSO_4$ and allowed to grow for one hour to a bacterial density of OD 595 (0.4 to 0.5) to ensure the log phase of the culture. The culture was chilled on ice, and the cells were centrifuged for 10 min at 2000 or 2500 rpm at 4°C. The cells were resuspended in 62 ml of ice cold 10 mM $MgSO_4$ (i.e one-half the culture volume) and the OD 595 was read and adjusted to 0.5 using cold 10 mM $MgSO_4$. The bacterial suspension was stored at 4°C for up to three weeks, however the highest plating efficiency was obtained with fresh cells (0-2 days old).

Testing the plating cells efficiency

One µl of the bacteriophage genomic library stock (2500 pfu) was diluted to 500 µl with SM solution. A control was prepared with no bacteriophage at all. Fresh PLK-17 cells (250 µl) were added to each of the library dilution tubes and incubated at 37°C for 20 min. One hundred µl of each culture were mixed with 3 ml of warm (45°C) 0.7% top agarose and transferred on top of room temperature 100 mm LB plates. The efficiency of the cells infection by the bacteriophage was measured in terms of plaque forming units.

Titration of the genomic library

A 10-fold serial dilution of bacteriophage stock was prepared in SM solution. The SM solution contained 5.8 g of NaCl, 2.0 g of $MgSO_4(7H_2O)$, 50 ml of 1 M Tris.HCl (pH 7.5) and 5 ml of 2 % gelatin per liter. The solution was autoclaved and stored at 4°C. Two sterile culture tubes were filled with 10 ml SM. Ten µl of the bacteriophage stock (2.4 x 10^6) were transferred into a first tube as a 10^{-3} dilution. Serial dilutions of the bacteriophage were performed: 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} and 10^{-8} and used to infect tubes containing 100 µl of PLK-17 cells. The dilutions were incubated at 37°C for 20 min to allow the bacteriophage particles to adsorb. Three ml of 0.7% warm agar or top agarose were transferred (one at a time) and poured on top of the LB plates avoiding air bubbles. The plates were swirled gently to allow distribution of the cells, allowed to solidify at room temperature for 10 min and incubated at 37°C. The formation of the plaques occurred at 8 hours, counted at 12 hours and the titration frequency was determined. The number of plates needed for screening the library was then determined based on the titration and the probability of finding the gene sequence in the library was calculated.

Plating the genomic library

Ten 150 ml LB plates were used to plate the library. Each plate contained 50,000 plaques. Twenty-one μl of the λ DASH II mouse library stock were used to inoculate 80 μl of SM solution. This mixture was performed in sterile capped tubes. Each of the tubes contained 500 μl of PLK-17 plating cells; these were incubated at 37°C for 20 min. Seven ml of 0.7% warm top agarose were added to each of the tubes (one at a time) and transferred quickly on top of the LB plates (Fig. 3.2). The plates were gently swirled and allowed to solidify at room temperature under the hood for 10 min and were incubated at 37°C. Plaques began forming about 8 hours and were counted after 12 hours. Nytran filters (137 mm Ø; 0.45 μ, Schleicher and Schüll, Keene, NH) were used for plaque replicas. The filters were initially autoclaved and identified asymmetrically by cuts at the edges. They were carefully placed on top of the library plates, avoiding bubbles, and carefully lifted.

Preparation of hybridization buffer

A total of 34 ml of deionized water were mixed with 100 ml deionized formamide, 2 ml 10% SDS, 50 ml of 20x SSPE, 4 ml of boiled herring sperm DNA and 5 ml of 100 x Denhardt's solution. This was mixed well and filtered using 0.2 μ filter. The necessary buffer was stored at room temperature for 2-3 days and the rest was stored at -20°C.

Radiolabeling the mouse Ahsg cDNA probe

The plaque filters obtained from the genomic library were screened using a

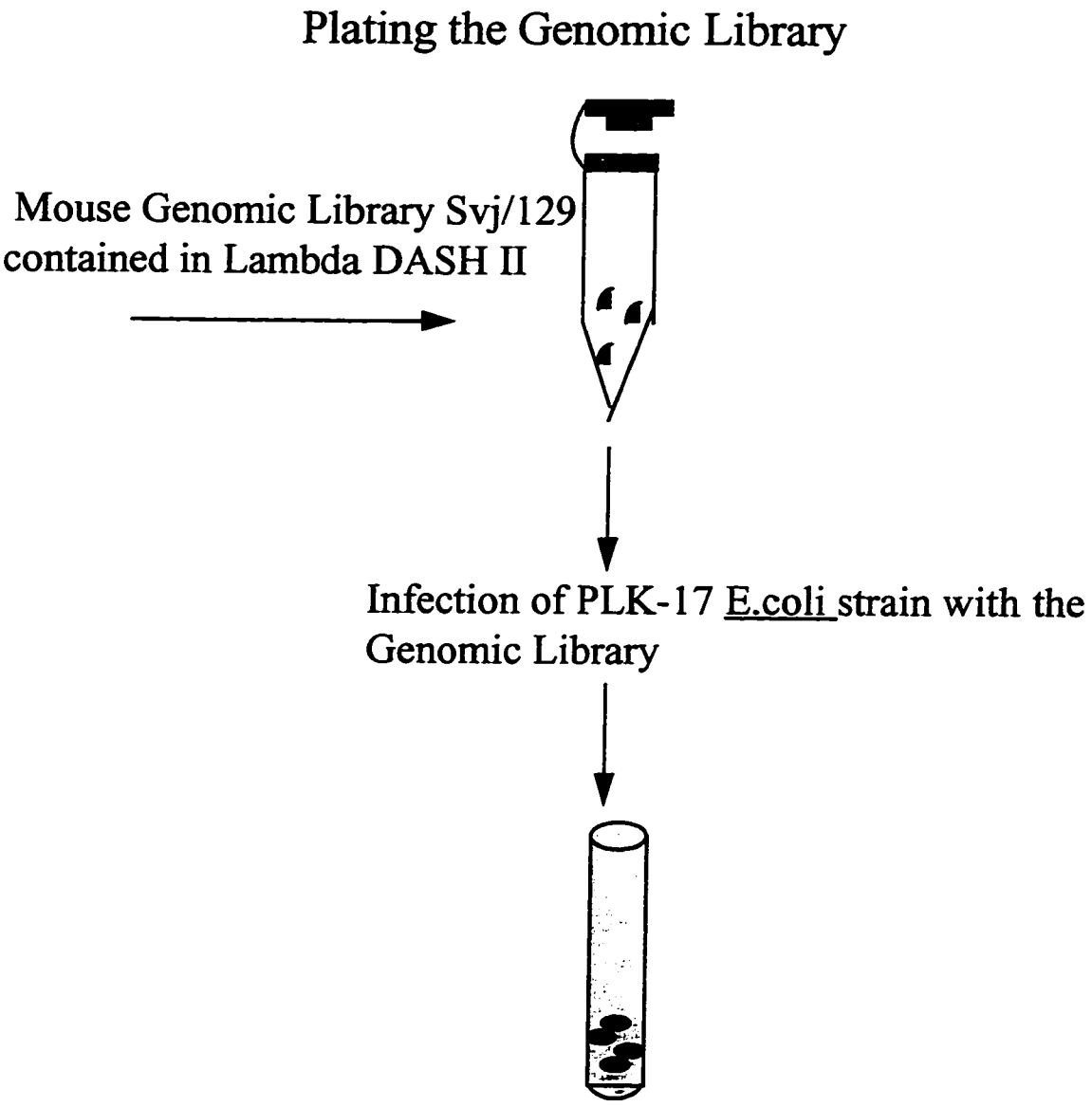


Fig. 3.2 Plating the genomic library. The mouse genomic library Svj/129 was contained in λ DASH II vector. The library was plated using *E. coli* strain PLK-17.

radiolabeled mouse cDNA probe (1.2 kb; 20-25 ng). The Prime-It kit (Stratagene, San Diego, CA) protocol was followed to prepare the radiolabeled probe. In this procedure random oligonucleotides were used as primers for labeling the cDNA. The primer template complex formed a substrate for the Klenow fragment of *E. coli* DNA polymerase I. This enzyme incorporated nucleotide monophosphates at the 3' end of the newly synthesized strand. The labeled DNA then was used as a hybridization probe to screen the genomic library. A total of 25 ng of DNA template were mixed with 0-23 µl of high quality water, and 10 µl of random oligonucleotide primers and added to a microfuge tube to a final volume of 34 µl. The DNA was denatured by heating at 98°C for 5 min and centrifuged briefly at room temperature. Then 10 µl of 5x primer buffer 5x dCTP were added, followed by 5 µl of labeled nucleotide [^{32}P] dCTP (300 Ci/mmole). A total of 1 µl of exo-deficient Klenow enzyme (5U/ml) was added to the tube and mixed. The entire reaction was incubated at 37°C for 10 min and stopped by adding 2 µl of "stop mix".

^{32}P-dNTP incorporation into the mouse Ahsg cDNA probe

The progress of the labeling reaction was measured by determining the proportion of radionucleotide incorporated into newly synthesized DNA. While, the probe was incubating, 1 µl aliquots were removed at 2, 5 and 10 min . The aliquots were diluted in 99 µl of 0.2 M EDTA. One µl of each dilution was spotted, in duplicate, onto Whatman DE 81 filter paper disks. The filters were dried for 15 min. Half of the filters were unwashed, and the other respective half was washed twice for 5 min at room temperature in 15 ml of 0.5 M Na_2HPO_4, water 3 times, and twice in 100 % ETOH (cold) and dried.

The unwashed filters were placed in counting vials with 5 ml scintillation cocktail, representing the total cpm. The treated filters represented the incorporated cpm which were also labeled on vials. A β-counter Ultima Gold was used to estimate the ratio of incorporation (washed halves) versus total unwashed.

Precipitation of the radioactive mouse Ahsg cDNA probe

To the tube containing the labelled reaction 1 µl of salmon sperm, 50 µl of 7.5 M NH_4Ac, and 125 µl of 100% ETOH were added, vortexed and incubated at -30°C for 30 min. The tube was then spun for 8 min at 12,000 rpm and the pellet allowed to dry. The DNA pellet was resuspend in 400 µl TE 1x, and kept on ice for 1 hour. The total reaction was added to a 15 ml tube and boiled for 5 min.

Genomic library filter processing and hybridization

Nytran filter replicas were processed in 0.5 N NaOH, 1.5 M NaCl 10 min, neutralized with 0.5 M Tris-HCl pH 8.0, 1.5 M NaCl 10 min, soaked in 2x SSC, 0.03 M NaCl, 0.03 M sodium citrate for 5 min, dried and crosslinked before being probed with mouse Ahsg cDNA (Fig. 3.3). A prehybridization step was carried out using 50% formamide, 5x SSPE, 10% Denhardt's, 0.1% SDS, 50 mg/ml denatured salmon sperm DNA (43°C, 20 min) before hybridizing in the same buffer, including the [^{32}P]-CTP labeled probe at 10^6 dpm/ml. The labeled probe was added to 10 ml of hybridization buffer with 0.5% SDS in a plastic bag which contained the nytran filters. The bag was sealed and incubated at 42°C by shaking, allowing the probe to hybridize with the Ahsg containing phage plaques.

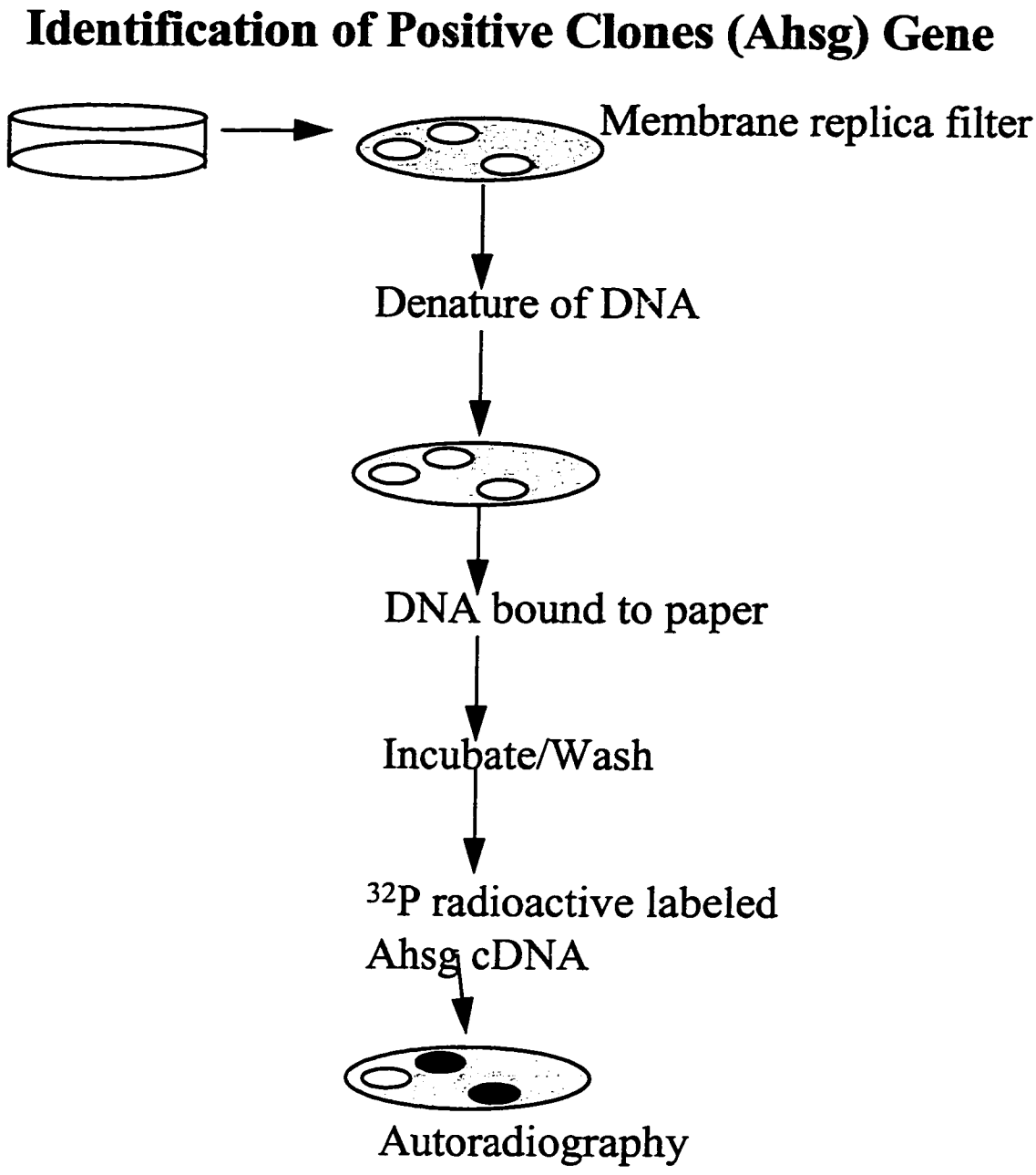


Fig. 3.3 Identification of positive clones Ahsg gene after high stringency hybridization. A total of 350,000 plaques were obtained by plating the genomic library. Nitrocellulose filters were used to perform replica plating of the plaques. The filters were processed,the DNA was bound, and the filters were washed. A radioactive mouse Ahsg cDNA was used as a probe to hybridize to the DNA on the membrane filters under high stringency. Plaques containing the genomic fragment of Ahsg were identified by autoradiography.

The filters were washed in 2x SSC and 0.5% SDS for 5 min, then in 2x SSC, 0.1% SDS solution for 10 min, and finally twice in 0.2x SSC, 0.5% SDS for 20 min, at 68°C.

Plaque purification and identification of positive clones bearing mouse Ahsg gene

Eleven positive plaques were rescreened, grown and the DNA isolated from 10 ml lysates using the Lambdasorb purification kit (Promega, Madison, WI). Restriction digestion was performed on one of these DNAs, λKOA-1B, using Bgl II and the fragments were separated and analyzed in a 1% ME agarose gel (Table 3.1). The fragments were subcloned into the vector pGEM4Z-BamHI site (Promega, Madison, WI). One hundred nanograms of pGEM4Z plasmid were digested with 0.65 µl of Bgl II enzyme (10U/µl) in the presence of 0.65 µl of 10x Universal buffer overnight at 37°C. Dephosphorylation, using 5 µl of calf intestinal phosphatase (CIP), was performed at 45°C for 20 min to prevent plasmid self-ligation. The ligation of the insert was performed using 2 µl of the CIP-BamHI pGEM4Z plasmid, 2 µl of 10x Universal buffer, 1 µl of T4 DNA ligase (12U/µl), 7.5 µl of water and 1 µl of Bgl II overnight at 12°C. A total of 1 µl of the ligation mixture was used to transform Xl-1 blue *E. coli* competent cells (Stratagene). Positive subclones were analyzed by restriction mapping and sequenced using primers SP6 and T7, in order to generate a putative map of the mouse Ahsg gene. An internal region of the gene was selected for further amplification by PCR, using primers to confirm the identity of the clones. The primers were designed based on the rat fetuin map (Falquerho *et al.*, 1991): KOAF1 5' GCCCTTCGGAGTGGTGTATGAGATG-3'OH and KOAB10 5'

ACGTTGGTATCGTTGAACGGAGTC 3'OH amplified a 984 bp product of the mouse Ahsg gene (Table 3.2, Table 3.3). Additional primers amplified exons 2, 3 and 4 of the mouse Ahsg. This entire sequence was obtained by superimposition of primers.

Table 3.1
Restriction digest of clone λKOA-1B

	λ BstEII	λ I857	λ KOA-1B	λ KOA-1B	λKOA-1B
DNA	10 µl	10 µl	4 µl	5 µl	5ul
10x buffer			3µl 1	1 µl	1 µl
enzyme			4 µl EcoRI	1 µl BglII	1 µl EcoRI
water			19 µl	3 µl	3 µl
Total			30 µl	10 µl	10 µl

A total of 40 µl of gel loading buffer mix were used per reaction.
The digested reactions were run in a 1% Sea kem agarose gel. Four µl of Ficoll were used in the final gel loading buffer mix.

Table 3.2
PCR reagents for amplification of the internal exons 2-4 in λKOA-1B

Reagents	Master mix used for λKOA-1B
25mM $MgCl_2$	4.0 µl
10xTaq Buffer	5.0 µl
2mM dNTP's	5.0 µl
10pM Primer KOAF1	0.8 µl
10pM Primer KOAB10	0.8 µl
Taq Pol **5U/ul**	0.4 µl
water	24 µl
Total	40 µl

A total of 5 µl of λKOA-1B was used in a volume of 20 µl of master mix reaction. A total of 20 µl of master mix and 5 µl of water was used as the control for the PCR reaction.

Table 3.3 PCR parameters for λKOA-1B amplification

Step	Time	Temp	Cycles
Hot start	5 min	94 °C	1x
Denaturation	45 sec	94 °C	31x
Annealing	25 sec	68 °C	
Extension	1 min 20 sec	72 °C	
Extension	10 min	72 °C	1x
Soak		4 °C	

Results

The PLK-17 cells used to plate the genomic library were tested for infection efficiency resulting in 333 plaque forming units. The final titration of the library resulted in 2.9×10^6 plaques per ml (Table 3.4). According to this titration 10 plates were needed each with 50,000 plaques in order to plate the complete library. The probability of having the mouse Ahsg gene in the library was calculated as follows:

$$N = \frac{\ln(1 - 0.99)}{\ln(1 - [2.0 \times 10^4 / 3.0 \times 10^9])}$$

$$= 6.9 \times 10^5$$

2.0×10^4 bp expected size of the gene

3.0×10^9 expected number of genes

Table 3.4
Titration of the genomic library

Dilutions	Number of plaques	Titration
2×10^{-4}	203	2.0×10^6
1×10^{-4}	293	2.9×10^6
1×10^{-5}	92	9.2×10^6
1×10^{-7}	0	0
1×10^{-8}	1	1

A radiolabeled mouse Ahsg cDNA probe (containing 71% radioactive incorporation of ^{32}P-dNTP) was used to screen the mouse genomic library. A total of 350,000 plaques of the λDASH2 Svj 129 genomic library were screened, resulting in 11 independent positive clones. The relatedness of these clones to the mouse Ahsg gene was confirmed by PCR amplification of crude phage DNA using mouse Ahsg primers

KOAF1 (5'-GCCCTTCGGAGTGGTGTATGAGATG-3'OH) and KOAB10 (5'-ACGTTGGTATCGTTGAACGGAGTC-3'OH) designed from targets in the cDNA sequence (Fig. 3.4). PCR amplification of the clones resulted in a fragment of 0.98 kb which could be cleaved into three pieces using BstE II digestion. One clone (λKOA-1B) was selected for further characterization (Fig. 3.5), and 10 μg of phage DNA prepared from plate lysates for restriction enzyme analysis. Restriction analysis performed on this clone using digestion with Eco RI or Bgl II (Fig. 3.6) suggested an insert size of 18.6-23.0 kb. Bgl II fragments of 6.0, 5.65, 3.45, 2.62 and 0.9 kb representing the mouse genomic insert were found (in addition to the λ arms); likewise, digest of λKOA-1B revealed Eco RI fragments of 6.6, 6.0, 4.85, 2.45, 1.7 and 1.42 kb (in addition to the λ arms). Two Bgl II fragments excised from an agarose gel (3.45 and 0.9 kb) were chosen for subcloning into the Bam HI site of pGEM4Z (Fig. 3.7), resulting in clones D and delta; both subclones were completely sequenced (Table 3.5; Fig. 3.8).

Sequence analysis of these two clones revealed that the smaller clone (delta) harbors the first exon (290 nt) in addition to 5'-regulatory elements up to the -154 position, as well as 431 nt of intron downstream of the first exon (Figs. 3.9 and 3.12). We suggest that position 155 in Fig. 3.10 be taken as the transcriptional start site (cap site) based on the alignment of expressed sequence tag (EST) clones available in public databases, especially those from the Sugano mouse liver EST project (A. Marra et al., Washington University, St. Louis, MO). Fig. 3.10 demonstrates the alignment of 14 of the more than 50 EST available sequences; the most 5' sequence (file identifier 1450748/ud65a11.y1, accession number AI047339) is taken to define the transcriptional start site. Upstream of the cap site (+1) can be found a TATA box (ATAAATT) at the –

24/-18 position, and two motifs suggestive of sites for transcription factors C/EBP-α (-58 to -45, CCTTTACGCAATTC) and HNF-3β (-126 to -115, ACTTATTTGCTT). Both of these factors are abundant in liver, and associated with the expression of liver-specific genes. Alignment of the corresponding human AHSG and rat AHSG upstream sequences (Fig. 3.14) revealed that all of these putative transcriptional elements are conserved in the proximal upstream regions of mouse, rat, and human genes. Moreover, this alignment of the three sequences revealed that the splice donor (SD) at the 3'-end of the first exon is precisely conserved among the three species, strongly suggesting that the mouse Ahsg genomic segment in clone delta represents the true ortholog of human AHSG. Beyond the SD site, there is little conservation of sequence between the mouse and human first intron.

The larger clone (D, 3.45 kb Bgl II insert) harbors exons 2, 3, and 4 (Fig. 3.11 and 3.13), in addition to two elements in the intron upstream of exon 2. The first of these elements is a 271 nt long microsatellite (T,C)n *(A,G)n composed of 96% C or T in the coding strand. The second element found in this intron is a 92 bp sequence with high homology to the family of B1 middle repetitive elements characteristic of mouse DNA (Quentin, 1989; Labuda *et al.*, 1991).

Discussion

The mouse clone we have isolated by screening a mouse Svj/129 genomic library (λKOA1B), harbored 18.6-23.0 kb of the mouse Ahsg gene. Sequence analysis showed that the mouse gene is organized with the same exon-intron organization as both the ratfetuin gene (Falquerho *et al.*, 1991) and the human AHSG gene (Osawa *et al.*, 1997).

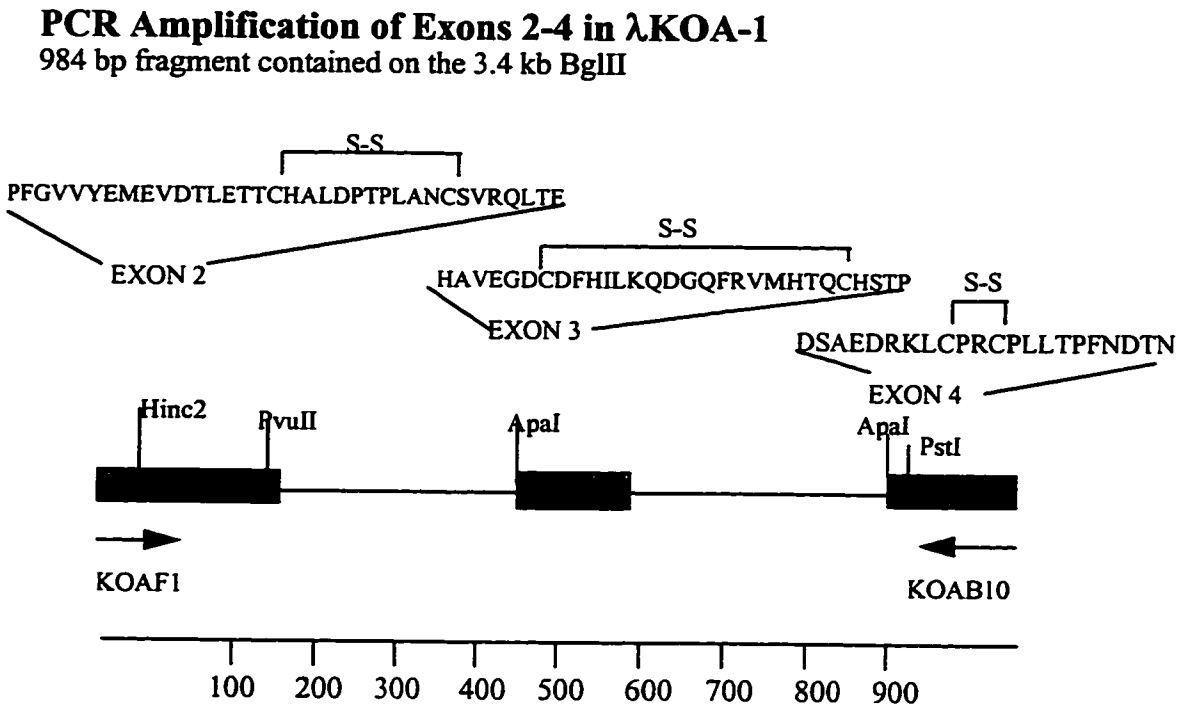


Fig. 3.4. PCR approach to recover a 984 bp of mouse Ahsg gene. The identification of mouse Ahsg gene was oncfirmed by PCR amplification of a 984 bp fragment using primers KOAF1 and KOAB10. This fragment was further analyzed by restriction mapping and revealed internal exons 2, 3 and 4 of mouse Ahsg gene.

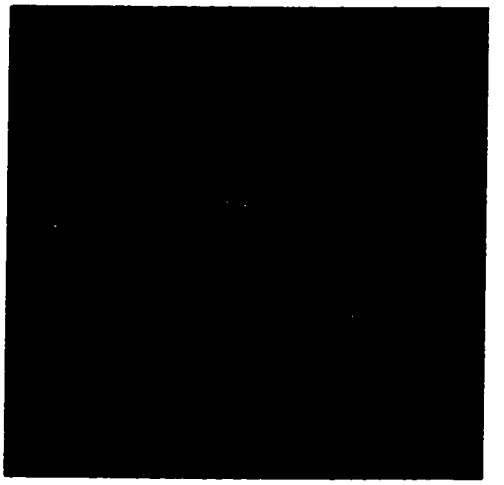
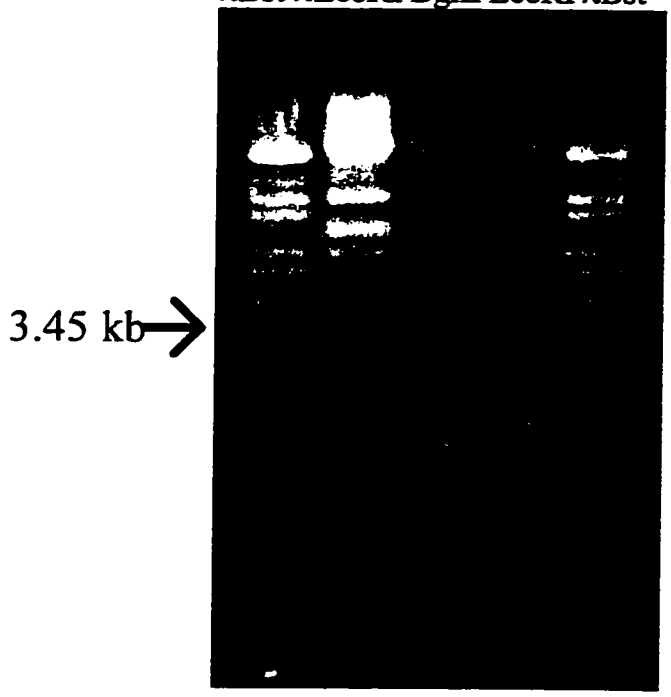


Fig. 3.5 Amplification of a positive genomic clone containing mouse Ahsg gene. One genomic clone λKOA-1B was identified and amplified. This clone contained the mouse Ahsg gene confirmed by restriction mapping and PCR of exons 2, 3 and 4. This figure represents the amplification of the pure clone λKOA-1B and the positive hybridization against radiolabeled mouse Ahsg cDNA.

Fig. 3.6. Restriction analysis of Ahsg genomic clone λKOA-1B. A 1.2 kb mouse Ahsg cDNA was used as a probe to screen a mouse genomic library. One of the eleven positive clones (λKOA-1B) was plaque-purified to homogeneity and 10 μg of DNA purified from plate lysates. DNA was cut with EcoRI or Bgl II and the DNA fragments separated on a 1% agarose gel. DNA in lanes 1 and 4 is a λBstE2 marker, lane 2 is λKOA-1B cut with Bgl II and lane 3 is λKOA-1B cut with EcoRI. Digestion with Bgl II generated bands ranging from 900 bp to about 8.5 kb. Two BglII fragments were selected for subcloning--a 3.45 kb BglII fragment (indicated by the arrow) and a 0.9 kb BglII band (not shown). Summation of the sizes of the non-vector fragments in lanes 2 and 3 imply that clone λKOA-1B, harbors a total of 18.6-23.0 kb of mouse DNA, more than twice the size of the known rat and human AHSG genes (7-8 kb; Falquerho *et al.*, 1991; Osawa *et al.*, 1997).

Clone λKOA-1

λBst λEcoRI BglII EcoRI λBst



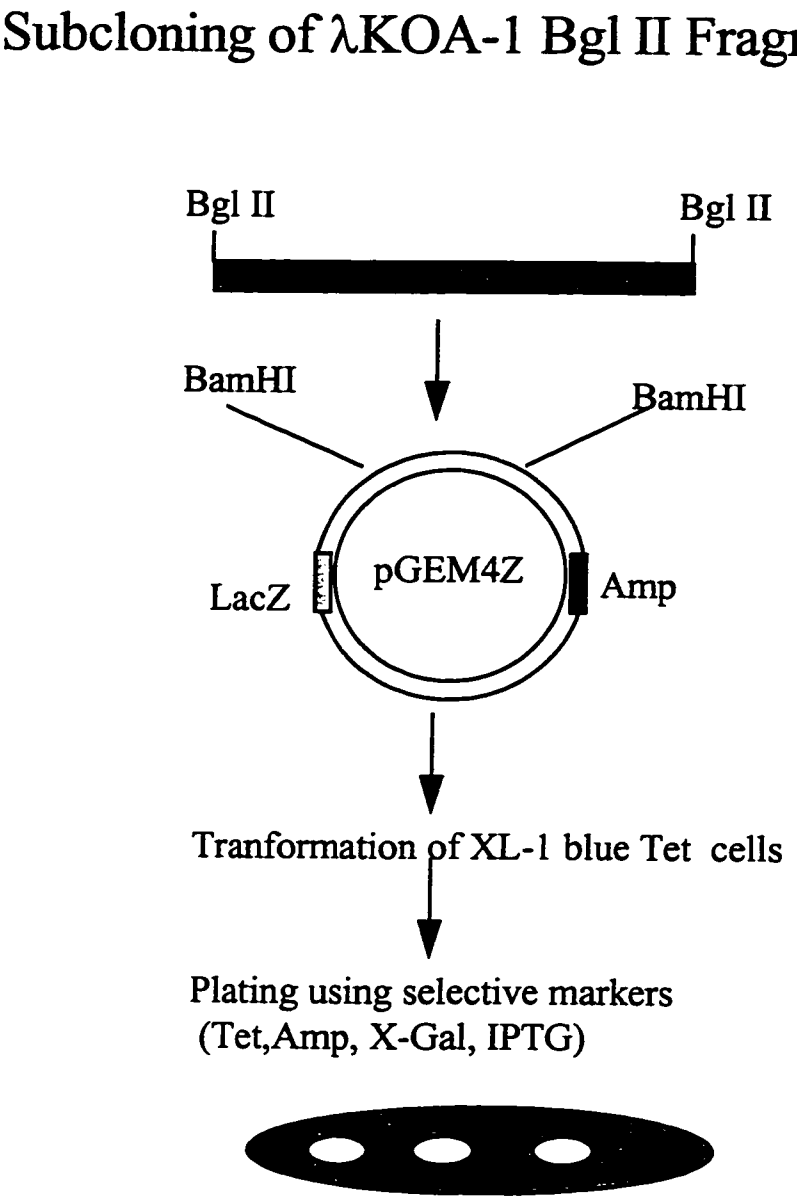


Fig. 3.7 Subcloning of λKOA-1B Bgl II fragments into pGEM4z plasmid vector. λKOA-1B positive genomic clone for mouse Ahsg gene was analyzed by restriction digest with Bgl II. Two fragments, a 3.45 kb and a 0.9 kb, were excised from an agarose gel and subcloned into pGEM4Z. These clones were named D and delta, respectively.

Table 3.5
List of primers used for sequencing the mouse Ahsg gene

Primer	Oligonucleotide	Sequence	Size bp
KOAF1	(putative exon 2) (111 bp)		
	5' GCCCTTCGGAGTGGTGTATGAGATG 3'	VC 0318 24	2014
		VZ 1021 23	
KOAB10	(putative exon 4) (164 bp)		
	5' ACGTTGGTATCGTTGAACGGAGTC 3'	VC 0430 11	1790
		VC 0318 25	1252
M13 reverse	5'AGCGGATAACAATTTCACACAGGA3'		
-40 17-mer	5'GTTTTCCCAGTCACGAC3'		
RSB1	5'TGCCAGCAGGTTGCATTTGGCTG3'		
RSF1	5'CAGCCAAATGCAACCTGCTGGCA3'		
RSF2	(exon 4)		
	5'GACTCCGTTCAACGATACCAACGT3'	VC0318 22	2078
		VC 0117 26	1938
SP6	5'ATTTAGGTGACACTATA3'	VC 0923 23	1900
		VC 0306 27	1854
		VC 0115 35	1406
SSF1	exon 1		
	5'GGATCCTGACATTTGCCCATTTTCC3'	VC 1211 28	
T7	5'CCCTATAGTGAGTCGTATTA3'	VC 0115 34	1280
VIV1	5' end ORF		
	5'CTGCCAATCCGCTCCACAAGGTA3'		
VIV2	3' end ORF		
	5'TGTGGTATTGCTTTGTCAGTGGA3'		
VIV3	intron 1, downstream Bgl II site near T7 end		
	5'GATCTCATATGCTAACAGACTGAAAC3'	VC 0318 23	2036
VIV4	200 bp downstream of Bgl II adjacent to SP6		
	5'TGCAAGCACAGCGCCTGGTGCAG3'	VC 0306 23	2096
VIV5	5'CATCTCATACACCACTCCGAAGGGC3'	VC 0328 26	1938
VIV6	5'AACATAGGTCAGCCACAGGCAGAAG3'		
VIV7	5'CTGTCCTGGAACTTACTATGTAG	VC 0408 35	2174
VIV8	(exon 3) (85 bp)		
	5'GACTTCCACATCCTGAAACAAGAC3'	VC 0416 12	2034
VIV9	exon 3		
	5'GTCTTGTTTCAGGATGTGGAAGTC	VC 0416 13	1968
VIV10	anneal AT 195 SEQ VC 040835		
	5'TGCTCAGAGTGAGGATTGACAAC3'		
VIV11	5'CATGGCACCTAGTGTGAACACTAG3'		
VIV11*	5'CCAATGCATGGCACCTAGTGTGAACAC3'		

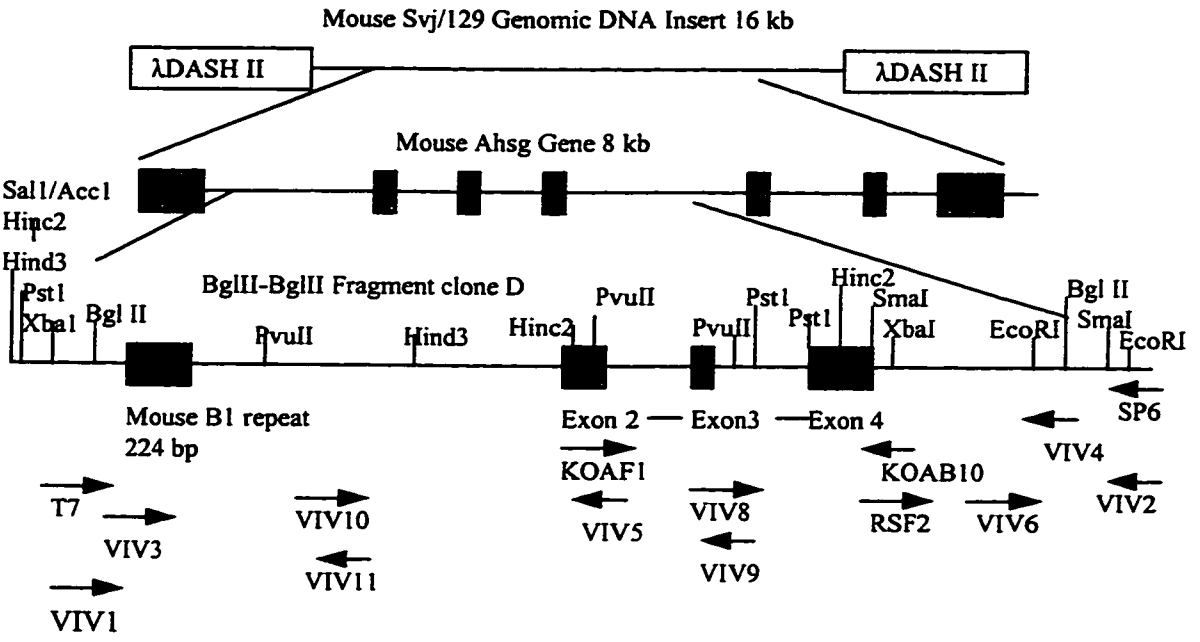


Fig. 3.8 Analysis of the 3.4 kb subclone by primer overlapping. Different primers were used to sequence the entire 3.4 kb Bgl II-Bgl II fragment. This fragment contains exons 2,3 and 4 of mouse Ahsg gene.

Fig. 3.9 Sequence of the 875 bp mouse Ahsg subclone delta. The 875 bp mouse Ahsg subclone *delta* contains 154 nt of 5'-flanking DNA in addition to exon 1 and part of the first intron. The DNA sequence analysis shown reveals a 154 bp segment upstream from the transcriptional start site, the first exon and part of the first intron. Putative transcriptional motifs [hepatic nuclear factor (HNF)-3β and C/EBP-α] are indicated, as well as the transcriptional start site. One of the primers used to construct the baculoviral cDNA expression clone (SSF1) is indicated. The DNA in exon 1 is indicated in uppercase. This sequence is available from GenBank, accession number AF025820.

Fig. 3.10 Mouse Ahsg deduced transcriptional start site. The transcriptional start site is deduced from the alignment of 14 EST clones available in the public database; the most 5' EST clone (AI047339; Sugano mouse liver EST project) is taken to define the transcriptional start site.

Fig. 3.9

```
   1 agatctgttg gggagagatg atgtcctaac ttatttgctt ttccagagct gctgtttgca
                                  HNF-3β
  61 aggattattt ggaaccagaa cagaaatcgt cccacgcctt tacgcaattc cttcggcggg
                                                c/EBP-α
 121 ctctgtcaga taaattaggc cctctgcccc tctattggtc tagctctcca agctgattat
             TATA box                          |---------- mRNA ---------->
                                              +1

 181 ccgggctgct cctgacattt gcccattttc cagggcctct ctggagcaac cATGAAGTCC
                        Primer SSF1 --->                      MetLysSer

 241 CTGGTCTTGC TCCTTTGTTT TGCTCAGCTC TGGGGCTGCC AATCCGCTCC ACAAGGTACA
     LeuValLeuL euLeuCysPh eAlaGlnLeu TrpGlyCysG lnSerAlaPr oGlnGlyThr

 301 GGACTGGGTT TTAGAGAATT GGCTTGTGAT GATCCAGAAG CGAAGCAAGT AGCTTTGTTG
     GlyLeuGlyP heArgGluLe uAlaCysAsp AspProGluA laLysGlnVa lAlaLeuLeu

 361 GCCGTGGACT ACCTCAATAA TCATCTTCTT CAGGGATTCA AACAGGTCTT GAATCAGATC
     AlaValAspT yrLeuAsnAs nHisLeuLeu GlnGlyPheL ysGlnValLe uAsnGlnIle

 421 GACAAAGTCA AGGTGTGGTC TCGGgtaagt gagcctacca ggaatgagct gaatgaatct
     AspLysValL ysValTrpSe rArg

 481 gggtagggga tctaacccag tgcctcaaag gctagcatct cccagtggag atggaatggc
 541 agcagagatt gggaaagaga gagcaggaga ggccacatca gtattgttgc cgtgtatctc
 601 acagaggatg ctcccacggg aagaggaagg gaccccgagg catagttcac agaccacagg
 661 cagaaggttg gacttgctgg gaaaacctgg gcccaatttg ctaatttgtg aatgacttct
 721 ctttcttgga ttacatttaa ctctgatgaa aaggaaagca gcatttgatc agtgaccatg
 781 tgtgacggcc atctaatgca gatcgtttca ttaaatcctc ccttcattca ttccagacct
 841 gcctgatagc catgttctcc ttagacaaca gatct
```

Fig. 3.10

```
TAAATTAGGC CCTCTGCCCC TCTATTGGTC TAGCTCTCCA AGCTGATTAT CCGGGCTGCT
   AI047339| ------ ---------- ---------- ----------
   AI046376|   -- ---------- ---------- ----------
   AA986200|    - ---------- ---------- ----------
   AA986067|      ---------- ---------- ----------
   AI048598|      ---------- ---------- ----------
   AI047875|      ---------- ---------- ----------
   AA987109|      ---------- ---------- ----------
   AI047382|       --------- ---------- ----------
   AA986257|       --------- ---------- ----------
   AI046467|       --------- ---------- ----------
   AI046373|       --------- ---------- ----------
   AI048620|       --------- ---------- ----------
   AI043154|       --------- ---------- ----------
   AI046480|        -------- ---------- ----------
```

Fig. 3.11 Sequence of internal exons 2-4 of mouse Ahsg gene. The 3.45 kb Bgl II fragment cloned pGEM4Z (clone D) was sequenced using a combination of dye primers (SP6 and T7, at the 3' and 5'-ends of the subclone, respectively), and dye terminator reactions. Sequencing primers are indicated beneath the DNA sequence. Exons are indicated in the DNA sequence as uppercase letters. The first intron contains 271 bp of the microsatellite (C,T)n*(A,G)n adjacent to a middle repetitive element of 92 bp in the B1 family. This sequence is available from GenBank, accession number AF025821.

```
   1 gatctcatat gctaacagac tgaaactatg aaaccgccaa tacttgtctg cttttctttt
         PRIMER VIV3 --->                                  |-- (CT)n ----

  61 cctgtttctg tctgtctgtc tgtctttctt cctttctttc tcttctctct ttctttctct
     ------------- microsatellite (CT)n repeat ----------------------

 121 tcctctttct ttcttcttcc ttcctttctc ttctttctct ttctttcttc ctttttcttc
     ------------- microsatellite (CT)n repeat ----------------------

 181 tttctttctc tttgctcttt ctttcccttc cttccttcct ttctcttctt tctcttctct
     ------------- microsatellite (CT)n repeat ----------------------

 241 ctttcttttc cttcctttct tccttccttc cttccttcct tccttccttc cttccttcct
     ------------- microsatellite (CT)n repeat ----------------------

 301 tccttctttc cttcttcttt ttgagacaga aaggtttctc tttgtagccc tgactgtcct
     -- (CT)n repeat -------|         |---- B1 repetitive element ----

 361 ggaacttact atgtagacca ggctggcctc aaacacacag agatccacct gcctctgcct
     Primer VIV7 -->
     ------------------------ B1 repetitive element ------------------

 421 cctggctcct ggaatcatag gcatgtgtca ccgactattt gtctgctttt aacatgcaaa
     -|

 481 gttggaaact ccatacggtt cagcttaaca taaagatgag aagaacaagt ttgtgtcact
 541 agagacttag gatttaggag gaaaataagg taaacaccag gatgctcaga gtgaggattg
 601 acaaccagct ttacaatggg acagctgatt tgaaaccacg gttttcctgg gtgagtttta
 661 agggcagttg gcaaaagacg taatggccgg ctctctgcct agtttacatg ctgaagggaa
 721 agccgtgagc gagcactgtg catgtgctac gtgctgattg tgagatgctc attatgggat
 781 gcccgagtgg atcaagaant tccctgcaca taaacccagt gcatctaccc atggttagtt
 841 ctgaggtctc cggagagtga aaatgcccag tgaactaaat tgggttgaga gttttcaaac
 901 tttggggcat ttcaaggtgt gaacggggaa tacatagaca ggtgaaacac tgaactcctc
 961 acagggtcct gcaagcttcc caaaatgctt ccatcctagt ggtgacagtt tcccagcctc
1021 agaatagaaa ggcggcaaac aggagatagg actctctgtg catccaggac ccaggaaggt
1081 agaagataaa gagccaaggg aggagcaaga gaaaccttta aggacacaaa cactcaaaga
1141 aagggagaaa agtgggcaac tagagagaag aaagaatgaa gcagaatgaa agatagcaaa
1201 gataataaac ctttcaagat aaaagctagc cctcagagtc acttctttgt aaagagagct
1261 cagaataagg agtatggctg ggacagctgt tggagcacgg ccccccctcc cgtccctttt
1321 ttcccccctcc cccctcccca tttcacaccc gctccatcta tagtggaaga ctaaaaagcc
1381 aaaacaaaac aaaaagaaaa aaaaaaaaaa ccactgcagc actgcatagc tggaaggggt
1441 ggggctgaca tctccttagt cctcccaccc ctcagccaag ccccaccaca gggctagtgt
1501 tcacactagg tgccatgcat tggatcagcg gagcagggcg tttgctcacc tctcccttct
     <-- Primer VIV11

1561 gtccggctcc acagCGGCCC TTCGGAGTGG TGTATGAGAT GGAAGTTGAC ACACTGGAGA
                  SA      Primer KOAF1 -->              exon 2
                      <--- Primer VIV5 ----------
                    ArgPro PheGlyValV alTyrGluMe tGluValAsp ThrLeuGluT

1621 CCACTTGCCA TGCTTTGGAC CCCACCCCGC TGGCAAACTG TTCTGTGAGG CAGCTGACTG
     hrThrCysHi sAlaLeuAsp ProThrProL euAlaAsnCy sSerValArg GlnLeuThrG

1681 AGCACgtgag tgctgccttg tggttggttg gtgggtgggt gggtgggtgg gagctgccca
     luHis

1741 gccaccacag ttcagcaaag tgcaggtttg gctttctcca tctcccagca gccatcttgg
1801 ctagccagag agcaaagtct aaaacccgct gtgggataga tggtgccttc cccgaggttg
1861 attttcacaa cacttgggct tttcttcttg aagccctcgg gagagcagat tatgatgttt
1921 caataacacc cgtgaaggtt gccttgggca ggttacctcc cacaaccctg ccaagacgct
1981 ccctgaatga gcagccagag tatatatact gcttcagaat gccggcatct gatttcttta
```

```
2041 cccagGCGGT GGAGGGAGAC TGTGACTTCC ACATCCTGAA ACAAGACGGC CAGTTCAGGG
                                  Primer VIV8 -------->     exon 3
          AlaVa lGluGlyAsp CysAspPheH isIleLeuLy sGlnAspGly GlnPheArgV

2101 TGATGCACAC CCAGTGTCAT TCCACCCCAG gtcagaaaac actgcctctt gtttttatct
     alMetHisTh rGlnCysHis SerThrPro

                                    SD
2161 cgtagaatga gaaaggaatc agaatagttt tgaactcaaa taggtctcac ttcctctgtg
2221 aggattctgg gtcctgggga ttctaatgcc atcttttaaa gaagcccgtt cttgtgggcg
2281 aacattgtcc ccgtggctgt gacttggtga ccttcataca gctgcttgaa ggcttagaga
2341 agaagtcatg ctacattgga gcactaggag ccctttctaa ataagcaagc tgttgtgagt


2401 acactggaga gctgcagttg agaccctgct atcttcccgc ccagACTCTG CAGAGGACGT
                                                   SA
                                                      SerA laGluAspVa

2461 TCGTAAGTTG TGCCCACGGT GCCCACTCCT GACTCCGTTC AACGATACCA ACGTGGTCCA
     lArgLysLeu CysProArgC ysProLeuLe uThrProPhe AsnAspThrA snValValHi

2521 CACCGTCAAC ACTGCCCTGG CTGCCTTCAA CACACAGAAT AATGGAACCT ATTTTAAACT
     sThrValAsn ThrAlaLeuA laAlaPheAs nThrGlnAsn AsnGlyThrT yrPheLysLe

2581 GGTGGAGATT TCCCGGGCTC AAAATGTGGt aaaaacttaa cactcttttg atagatttgg
                                   SD
     uValGluIleSerArgAlaGlnAsnValCy

2641 gcgatttggt ggcccttggg gcatgtgtgg gggtgataac cagaagaaaa ggaaacattg
2701 gctggaagaa ctggcagggg ttctagaact tatggagccc taaactctca gcagcgctgt
2761 cccaaactga gccagttcaa cataggtcag ccacaggcag aaggcaggta acaccctggc
2821 ctcctggctt tacctaacac ttaatagcag ggctctctgt tcagacacaa tacattcacc
2881 gggtgccaca cgtttacacc ctgccagtaa catctgccgc agtctgggaa tcaacactaa
2941 caaaggtatg ggcaaatact ggaaggttcc taatctgcct ttcaaatcca ggtttttgag
3001 gtgggagggg accatctaat tgtatagcca aagcaactat ttgagtgcaa tagacttgag
3061 atgtttaagg aagctggcaa tggaaataag tcaagacata cttgcaaata cattagtgta
3121 ggtggtgatt ctgtaattcc tgggacaatt cccatcccat actgcaccag gcgctgtgct
                                                <--- Primer VIV4 -------

3181 tgcaaggctc ccagcgtcag ggggaaggaa gcacagtgac ttccattttg atcctgctgt
3241 gggaaactgg ggtgggggca tcttttcact tcccgcttcg agcctgggat gactggaaca
3301 ttgaattctg aaggttgagg gccaggagat gctgggtttc catccctgcc ggactaaagt
3361 tagccttttg gcttcctgtt tcctctctga aaacttaacc tctgccccat gcgatggaca
3421 acgatctctt  gccaatactt tgagacgact  cagatc
```

Fig. 3.12 Schematic structure of subclone delta. This figure represents the schematic structure of the upstream subclone delta whose sequence is shown in Fig. 3.9. Putative transcriptional motifs (hepatic nuclear factor (HNF)-3β and C/EBP-α) are indicated, as well as the transcriptional start site. The segment of the first exon which encodes the first 85 amino acids of mouse α_2-HS-glycoprotein is shown in the shaded box.

Fig. 3.13 Schematic structure of subclone D, exons 2-4. This figure represents the schematic structure of exons 2, 3, and 4, including part of the first intron and part of the intron downstream from exon 4. Shaded boxes indicate the exons. The 271 bp microsatellite (C,T)n*(A,G)n and the 92 bp middle repetitive (B1 family) element are indicated by bracketing.

Fig. 3.12
Ahsg proximal promoter region and exon 1

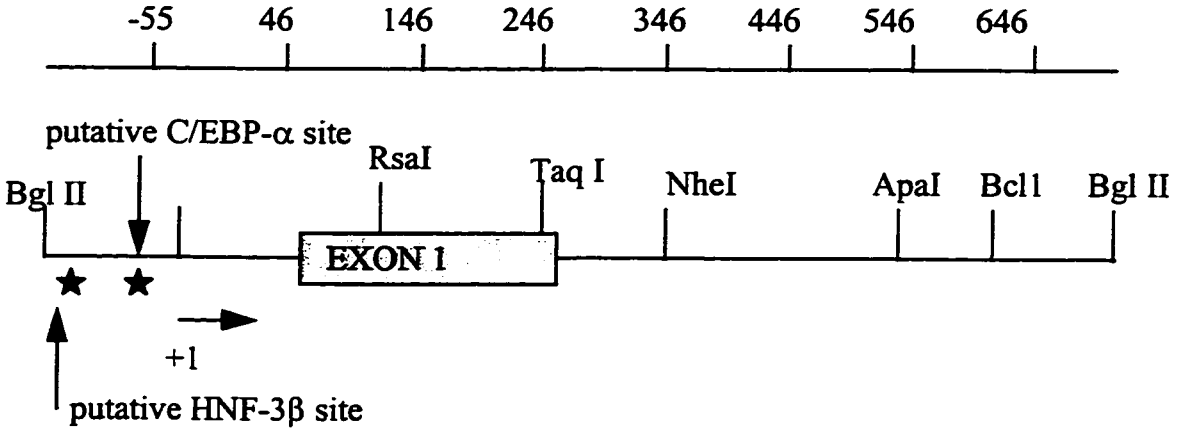


Fig. 3.13
Ahsg gene, exons 2-4

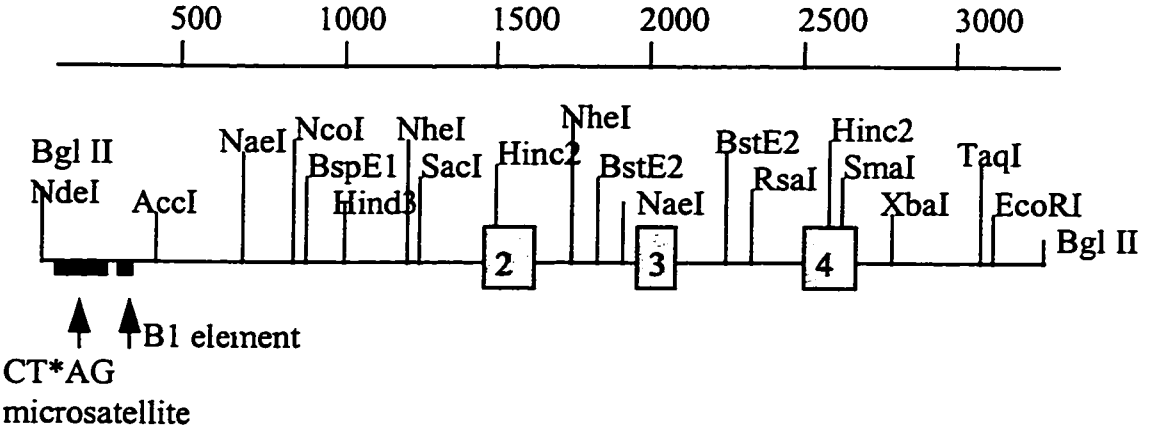
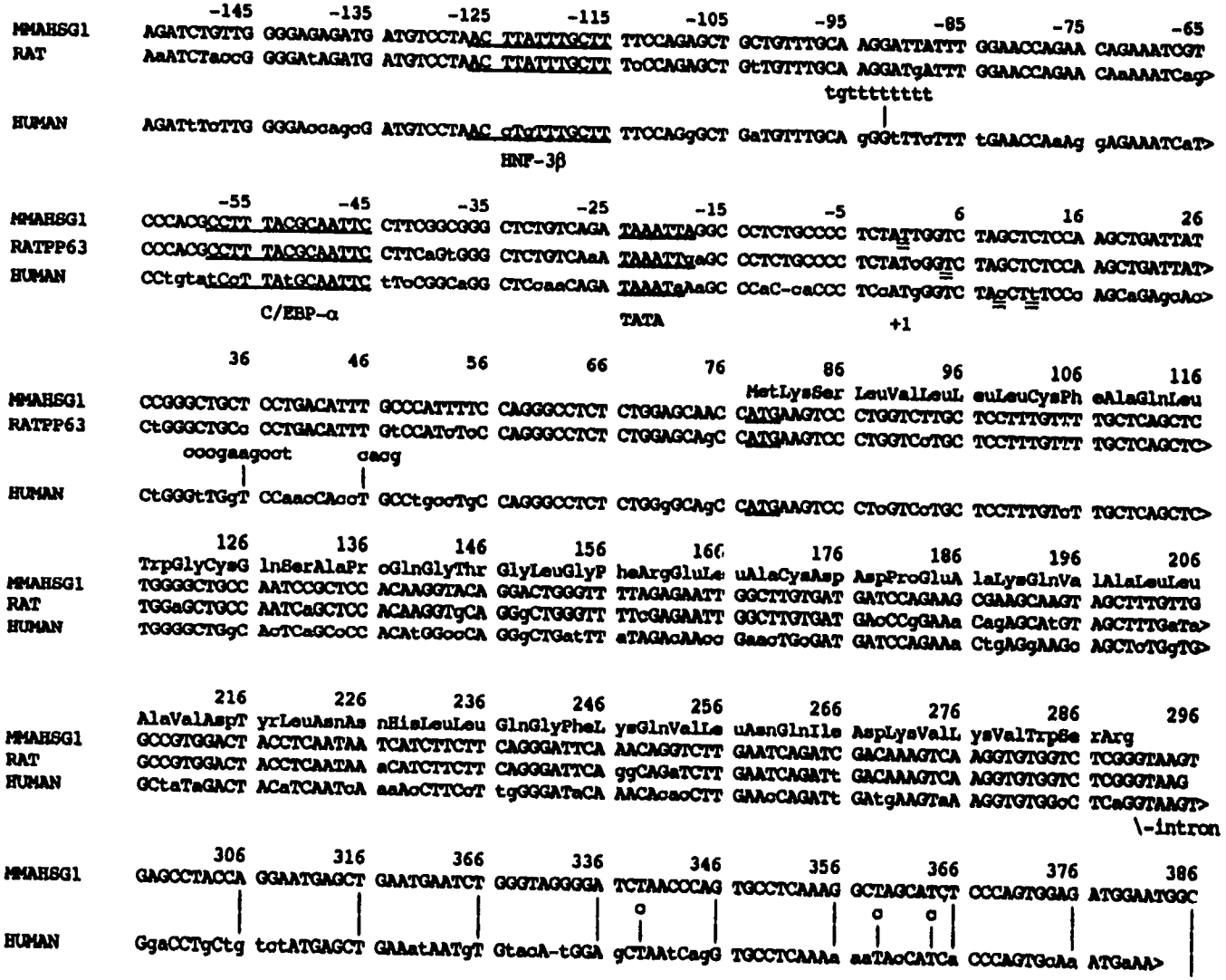


Fig. 3.14 Alignment of the proximal promoter sequences of mouse, rat, and human AHSG genes reveals an evolutionary conservation of putative transcription factor motifs, TATA box, and splice donor (SD) sequences. The mouse sequence is taken from Fig. 3.8; the rat sequence is taken from GenBank files M36547 (Falquerho *et al.*, 1991) and X63446 (Rauth *et al.*, 1992); the human sequence is taken from GenBank files Y09540 (Banine *et al.*, 1998) and M16961 (Lee *et al.*, 1987). Sequences were aligned using CLUSTAL. Transcriptional start site are indicated by double underlining. The C/EBP-α and HNF-3β motifs and the MET initiator codon are indicated by single underlining. The conceptual translation of the mouse Ahsg sequence is indicated above the line MMAHSG1. Where the rat or human sequence agrees with the nucleotide in the mouse sequence, it is capitalized; otherwise, mismatched bases are indicated in lowercase in the human and rat sequence lines. The human sequence has two insertions relative to the mouse sequence; the rat sequence has a single deletion relative to the mouse sequence, filling in the gap with a (-). The numbering above the mouse sequence line is relative to the mouse transcriptional start site defined in Fig. 3.9.


-145 -135 -125 -115 -105 -95 -85 -75 -65
MMAHSG1 AGATCTGTTG GGGAGAGATG ATGTCCTAAC TTATTTGCTT TTCCAGAGCT GCTGTTTGCA AGGATTATTT GGAACCAGAA CAGAAATCGT
RAT AaATCTaccG GGGAtAGATG ATGTCCTAAC TTATTTGCTT ToCCAGAGCT GtTGTTTGCA AGGATgATTT GGAACCAGAA CAaAAATCag>
tgttttttt
HUMAN AGATTtToTTG GGGAocagcG ATGTCCTAAC gTgTTTGCTT TTCCAGgGCT GaTGTTTGCA gGGtTToTTT tGAACCAaAg gAGAAATCaT>
HNF-3β
-55 -45 -35 -25 -15 -5 6 16 26
MMAHSG1 CCCACGCCTT TACGCAATTC CTTCGGCGGG CTCTGTCAGA TAAATTAGGC CCTCTGCCCC TCTATTGGTC TAGCTCTCCA AGCTGATTAT
RATPP63 CCCACGCCTT TACGCAATTC CTTCaGtGGG CTCTGTCAaA TAAATTaGC CCTCTGCCCC TCTATaGGTC TAGCTCTCCA AGCTGATTAT
HUMAN CCtgtatCoT TAtGCAATTC tToCGGCaGG CTCoaaCAGA TAAATaAaGC CCaC-caCCC TCoATgGGTC TAgCTtTCCa AGCaGAgoAo>
C/EBP-α TATA +1
36 46 56 66 76 86 96 106 116
MetLysSer
MMAHSG1 CCGGGCTGCT CCTGACATTT GCCCATTTTC CAGGGCCTCT CTGGAGCAAC CATGAAGTCC CTGGTCTTGC TCCTTTGTTT TGCTCAGCTC
RATPP63 CtGGGCTGCo CCTGACATTT GtCCATaToC CAGGGCCTCT CTGGAGCAgC CATGAAGTCC CTGGTCoTGC TCCTTTGTTT TGCTCAGCTC>
ooogaagoot cacg
HUMAN CtGGGtTGgT CCaacCAccT GCCtgooTgC CAGGGCCTCT CTGGgGCAgC CATGAAGTCC CToGTCoTGC TCCTTTGToT TGCTCAGCTC>
126 136 146 156 166 176 186 196 206
TrpGlyCysG lnSerAlaPr oGlnGlyThr GlyLeuGlyP heArgGluLe uAlaCysAsp AspProGluA laLysGlnVa lAlaLeuLeu
MMAHSG1 TGGGGCTGCC AATCCGCTCC ACAAGGTACA GGACTGGGTT TTAGAGAATT GGCTTGTGAT GATCCAGAAG CGAAGCAAGT AGCTTTGTTG
RAT TGGaGCTGCC AATCaGCTCC ACAAGGTgCA GGgCTGGGTT TToGAGAATT GGCTTGTGAT GAoCCgGAAa CagAGCAtGT AGCTTTGaTa>
HUMAN TGGGGCTGgC AcTCaGCoCC ACAtGGooCA GGgCTGatTT aTAGAoAAoo GaaoTGoGAT GATCCAGAAa CtgAGgAAGo AGCToTGgTG>
216 226 236 246 256 266 276 286 296
AlaValAspT yrLeuAsnAs nHisLeuLeu GlnGlyPheL ysGlnValLe uAsnGlnIle AspLysValL ysValTrpSe rArg
MMAHSG1 GCCGTGGACT ACCTCAATAA TCATCTTCTT CAGGGATTCA AACAGGTCTT GAATCAGATC GACAAAGTCA AGGTGTGGTC TCGGGTAAGT
RAT GCCGTGGACT ACCTCAATAA aCATCTTCTT CAGGGATTCA ggCAGaTCTT GAATCAGATt GACAAAGTCA AGGTGTGGTC TCGGGTAAG
HUMAN GCtaTaGACT ACaTCAATCA aaAoCTTCoT tgGGGATaCA AACAcaoCTT GAAoCAGATt GAtgAAGTaA AGGTGTGGoC TCaGGTAAGT>
\-intron
306 316 366 336 346 356 366 376 386
MMAHSG1 GAGCCTACCA GGAATGAGCT GAATGAATCT GGGTAGGGGA TCTAACCCAG TGCCTCAAAG GCTAGCATCT CCCAGTGGAG ATGGAATGGC
HUMAN GgaCCTgCtg tctATGAGCT GAAatAATgT GtaoA-tGGA gCTAAtCagG TGCCTCAAAa aaTAoCATCa CCCAGTGcAa ATGaAA>

Both the rat fetuin gene and the human AHSG gene feature 7 exons. The genomic subclones we have sequenced cover exon 1 (Figures 3.9 and 3.12) and exons 2-4 (Figs. 3.11 and 3.13). We have not sequenced clone λKOA-1B downstream of exon 4, so we are unable to address the question of exon-intron organization downstream of exon 4. In all three species, intron 1 occurs at the same codon position and features the same splice acceptor (SA). The sequence data presented in this study showed that intron 1 is 2000 bp in length in the mouse, slightly larger than the 1.7 kb reported for the corresponding intron in the rat (Falquerho *et al.*, 1991), and slightly smaller that the corresponding intron in the human gene (2.3 kb; Osawa *et al.* 1997). Clone λKOA-1B also contains a 271 bp tract of (T,C)n *(A,G)n adjacent to a B1 element of 92 bp. Numerous copies of B1, B2 and D1 elements are found in rodent genomes (Quentin *et al.*, 1989; Labuda *et al.*, 1991). Some of these B1 elements are not neutral relics of evolutionary spread of the DNA element, but may play a functional role in the regulation of the genes in which they are located. For example, B1 elements can act as negative regulators of gene expression (Saksela *et al.*, 1993; Winoto *et al.*, 1989). Moreover, androgen regulation of one gene seems to have been acquired during evolution through the insertion of a B1 repetitive element into the transcription unit (King *et al.*, 1986). Whether the B1 element in the first mouse Ahsg intron plays a role in the regulation of Ahsg transcription has not yet been addressed experimentally. The conservation of C/EBP-α and HNF-3β binding sites in the proximal 5' upstream region of the mouse and human AHSG gene is likely to have a functional significance. Falquerho *et al.* (1992) have shown these motifs to be functional in transient transfection of the rat AHSG gene. Moreover, Banine *et al.*, (1998) have analyzed the human AHSG promoter-enhancer in transient transfection

assays as well, these studies suggested a functional regulatory role for the C/EBP-α and HNF-3β sites in the human gene. The putative HNF-3β site in the mouse Ahsg gene is conserved between mouse, rat and human. Given the role of human (Srinivas *et al.*, 1993), rat (Auberger *et al.*, 1989), and mouse (this study) proteins in the inhibition of insulin receptor function, it is intriguing to note that one form of maturity onset diabetes of the young (MODY), maps to the gene encoding HNF-3β in man (Hani *et al.*, 1998; Lindner *et al.*, 1997; Kaisaki *et al.*, 1997). Another site conserved between the rat, mouse, and human genes is the putative C/EBP-α site at -58 to –45 (CCTTTACGCAATTCC in mouse). This site has been implicated in the cytokine-induced down regulation of the rat fetuin gene associated with the acute phase reaction (Akhoundi *et al.*, 1994).

CHAPTER IV

Chromosomal mapping of mouse Ahsg

Introduction

The proposed studies were performed in order to identify the mouse Ahsg gene chromosomal location. The 3' end of the mouse Ahsg cDNA was used as the target region for PCR amplification. This region was selected to increase the possibility of finding sequence polymorphism between different mouse strains. Two primers were designed based on this region and used for the analysis of the mouse Ahsg gene mapping. A PCR was performed using these two primers in a mouse interspecific backcross panels (BSS) C57BL/6J and *Mus Spretus* from 96 animals. The PCR amplifications were ran in a SDS-PAGE gel and the alleles types were analyzed by Map Manager computer program.

This study is the first to identify the mouse Ahsg gene location at 16 cM at chromosome 16. This position is syntenic to the human AHSG at chromosome 3q27 providing another evidence that mouse Ahsg is the ortholog of the human gene.

Material and Methods

Primer design and PCR conditions

Using the 3'-UTR of the mouse Ahsg cDNA as a target, a primer pair [sense 5'-CTTCAAAATCTAGGCTTGATTCGG-3'OH and antisense 5'-GCTTTATGCCTTTCATCAAATTTGACCATT-3'OH] was selected using the Primer Detective program (Lowe *et al.*, 1990). Mouse genomic DNA (25 ng) was amplified

using the above primers in a Thermal Cycler Model 9600 (Perkin-Elmer-Cetus, USA). The reaction contained 25 mM $MgCl_2$, 10x buffer, 2.5 mM each d NTP's, 0.1 mM primer (sense), 0.1 mM primer (antisense), and 0.65 units Taq polymerase (Perkin-Elmer-Cetus, USA). The samples were heated at 95°C for 1 hour and amplified by 35 cycles of denaturation (94°C for 30 sec), annealing (at the optimized temperature of 58°C for 30 sec), and extension (at 70°C for 1 min), followed by 3 min of extension (70°C) after the last cycle.

Typing of the interspecific backcross mouse DNA's

The Jackson Laboratory BSS interspecific backcross mouse panels were used to determine the mouse Ahsg chromosomal location (Rowe *et al.*, 1994). BSS panel consisted of one C57BL/6J and one *M. spretus* as controls, and 94 animals that were obtained by crossing the (C57BL/6J x *M. spretus*) F1 females with C57BL/6J males. For each marker, the following reagents were combined in a total of 1430 µl mixture: 130 µl of 10x PCR buffer, 78 µl of 25 mM $MgCl_2$, 104 µl of 2.5 mM dNTP's, 65 µl of 0.1 mM stock primers (sense and antisense), 8.45 Units of Taq polymerase, and distilled water. Five microliter aliquots were dispensed to each 0.5 ml PCR reaction tube in 96 well format using a single-channel electronic micropipetter (Proline Electronic, Finland). The genomic DNA from 96 animals (5 µl each from the BSS contained in 96 well microtiter plates at 5 ng/ml) was transferred to each PCR tubes. This process was performed manually with an 8 channel micropipetter (Flow Laboratories, USA) followed by the automated system Biomek 1000 (Beckman, USA). The PCR reactions were performed under the same conditions described above.

Polyacrylamide gel electrophoresis

A customized polyacrylamide gel electrophoresis apparatus (Nihin Eido, Japan) was used to obtain high resolution mapping results. A 28-well comb was specially designed to accommodate two interdigitated sample loadings with a 12-channel micropipetter. A total of 24 samples were loaded per 10% gel. The gels were run at 250 volts for 1 hour, stained with 0.5 µg/ml of ethidium bromide and photographed by UV transillumination.

Mapping analysis

Allele types, C57Bl/6J or *M. spretus*, were scored by visual inspection of the gels and analyzed with the computer program, Map Manager (Manly *et al.*, 1993). The localization of the markers was determined according to the composite map of backcross panels (Rowe *et al*, 1994). The composite map of the BSS panel data contained 451 markers, including 49 MIT markers. In these composite maps, the average centimorgan length of the 95 % confidence interval for these markers is 7.6 cM (BSS).

Results

In order to map the mouse Ahsg gene to its chromosomal site, we targeted a PCR amplicon of 198 bp located in the 3'-UTR of the mouse Ahsg cDNA (Takahashi and Ko, 1994) Fig 4.1. The data indicated a localization of the Ahsg gene to chromosome 16 at approximately 16 cM, adjacent to Dagk3 (Diacylglycerol 3-kinase) gene, a position syntenic with human chromosome 3q27, the location of the human AHSG gene (Lee *et al.*, 1987; Rizzu and Baldini, 1995). The mapping profile of the Ahsg gene, with respect

to the genes already mapped on chromosome 16, and a composite map of the Jackson BSS panel Map, versus the MGD composite map are shown in (Fig. 4.2; Table 4.1).

Discussion

We have successfully employed the 3' UTR of the mouse Ahsg cDNA as a target for interspecies polymorphism. The 3' UTR region was chosen because it is not disrupted by introns in the human gene (Osawa *et al.*, 1997), and, therefore, the primer pairs designed from the 3' short end of the cDNA should amplify the same 198 bp fragment from the genomic DNA. The 3' UTR sequences constitute a rich source of genetic markers for the mouse genome (Takahashi and Ko, 1993). Using this PCR based mapping technique, and panels between C57BL/6J and *Mus spretus,* the mouse Ahsg gene was localized to chromosome 16 at 16 cM. This location is synthenic with the position of the human AHSG gene on chromosome 3 band q27. It is interesting to note that the mouse Ahsg maps in the vicinity of other genes implicated in signal transduction and gene regulation such as Dagk3 (diacylglycerol 3-kinase, gamma 3, 15.5 cM), Prkm1 (protein kinase, mitogen activated kinase 1, 14.5 cM), and EIF4β, eukaryotic translation initiation factor 4β, 14.2 cM). These genes have also been mapped at the syntenic position in man.

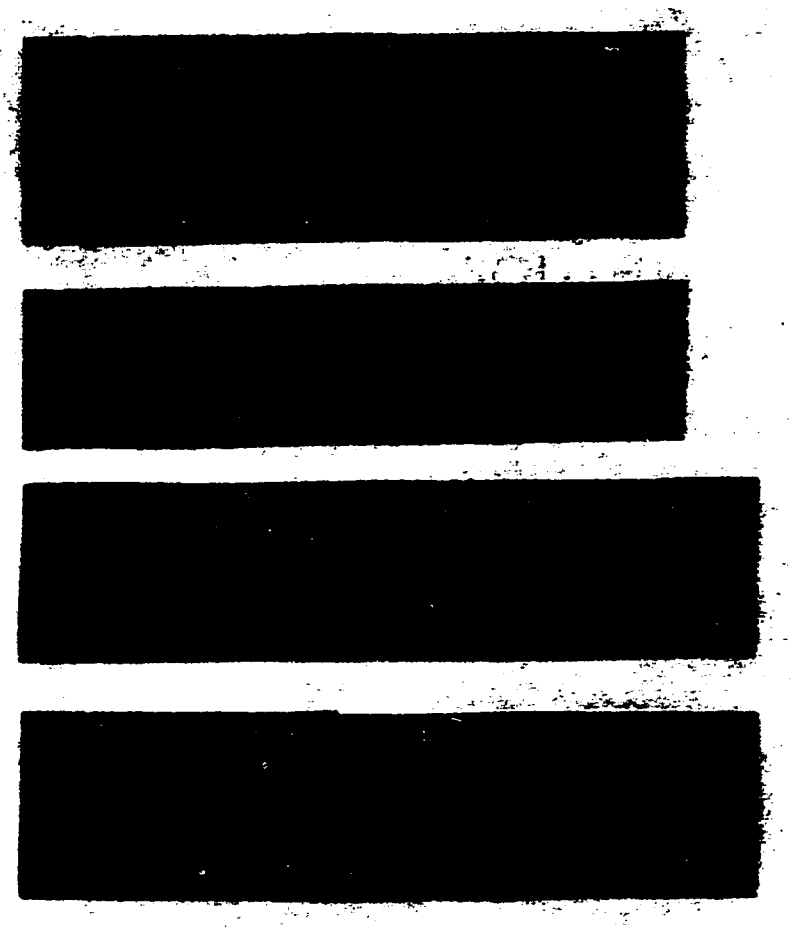
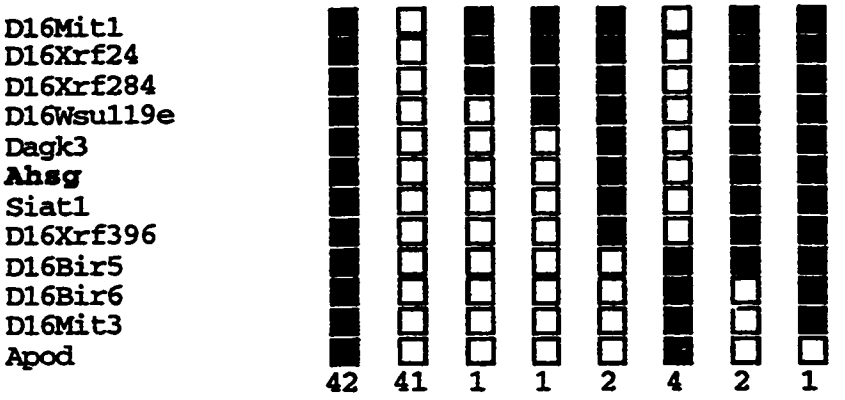


Fig. 4.1 Mapping profile of Ahsg gene. Two primers were engineered to amplify 198 bp at the 3'UTR of the mouse Ahsg cDNA. This site was used to increase the probability of finding polymorphism between different mouse strains. The PCR revealed two different patterns, B or S. The analysis of the data was performed by a computer program which compares the data to a Jackson laboratory BSS panel. According to the data a composite map was created which assigned the location of mouse Ahsg to chromosome 16.

Fig. 4.2 Composite map of the Jackson BSS panel versus MGD map. Chromosomal mapping of the mouse Ahsg gene obtained from typing patterns derived from PCR analysis using the Jackson Laboratories BSS interspecific backcross. Locations of allele types of C57B1/6J or *M.spretus* were determined by the composite map of backcross panels. Mouse Ahsg is mapped to chromosome 16 at 16 centimorgans. Panel A shows the mapping profile of the 96 animals used and the location of mouse Ahsg with respect to other genes mapped to chromosome 16. Demonstration of a composite map of the Jackson BSS panel Map versus the MGD composite map. The Ahsg gene is localized at approximately 16 cM closely to the Dagk3 gene (Panel B).

A


D16Mit1
D16Xrf24
D16Xrf284
D16Wsu119e
Dagk3
Ahsg
Siat1
D16Xrf396
D16Bir5
D16Bir6
D16Mit3
Apod
42
41
1
1
2
4
2
1

B

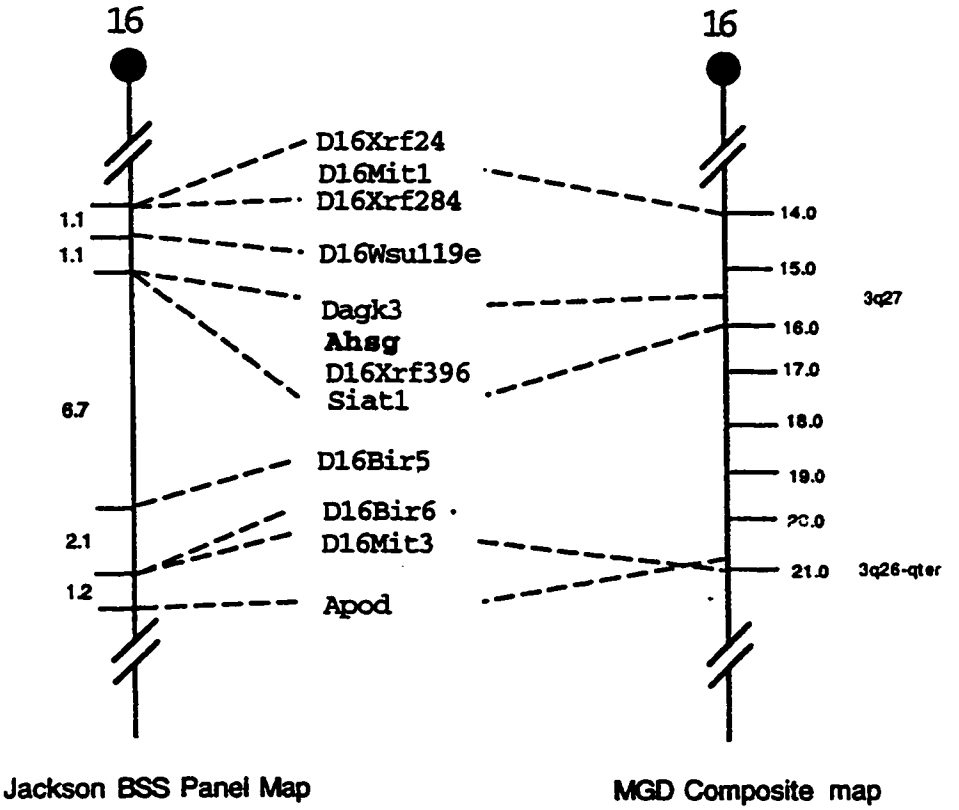

16
16
D16Xrf24
D16Mit1
D16Xrf284
1.1
1.1
D16Wsu119e
Dagk3
Ahsg
D16Xrf396
Siat1
6.7
D16Bir5
D16Bir6
D16Mit3
2.1
1.2
Apod
14.0
15.0
3q27
16.0
17.0
18.0
19.0
20.0
21.0
3q26-qter
Jackson BSS Panel Map
MGD Composite map

Table 4.1 Genes located in the vicinity of Ahsg at chromosome 16

Chromosome	*cM Position*	*Band*	*Symbol, Name*
16	14.0		*D16Mit1*, DNA segment, Chr 16, Massachusetts Institute of Technology 1
16	14.0		*D16Mit209*, DNA segment, Chr 16, Massachusetts Institute of Technology 209
16	14.0		*D16Mit35*, DNA segment, Chr 16, Massachusetts Institute of Technology 35
16	14.0		*D16Mit98*, DNA segment, Chr 16, Massachusetts Institute of Technology 98
16	14.0		*D16Sel1*, DNA segment, Chr 16, Seldin 1
16	14.0		*D16Xrf24*, DNA segment, Chr 16, XREFdb 24
16	14.1		*D16Mit2*, DNA segment, Chr 16, Massachusetts Institute of Technology 2
16	14.2		*D16Xrf284*, DNA segment, Chr 16, XREFdb 284
16	14.2		*Eif4b*, eukaryotic translation initiation factor 4B
16	14.25		*Iapls1-12*, intracisternal A particle, lymphocyte specific 1-12
16	14.25		*Iapls3-6*, intracisternal A particle, lymphocyte specific 3-6
16	14.5		*Prkm1*, protein kinase, mitogen activated kinase 1
16	15.5		*Dagk3*, diacylglycerol kinase , gamma 3
16	**16.0**		**Ahsg, α_2HS-glycoprotein**
16	16.0		*Clc2*, chloride channel 2
16	16.0		*Dyl3*, dishevelled 3, dsh homolog (Drosophila)
16	16.4		*D16Ncvs1*, DNA segment, Chr 16, National Cardiovascular Center, Shionogi 1
16	16.4		*D16Ncvs10*, DNA segment, Chr 16, National Cardiovascular Center, Shionogi 10
16	16.4		*D16Ncvs11*, DNA segment, Chr 16, National Cardiovascular Center, Shionogi 11
16	16.4		*D16Ncvs12*, DNA segment, Chr 16, National Cardiovascular Center, Shionogi 12
16	16.4		*D16Ncvs13*, DNA segment, Chr 16, National Cardiovascular Center, Shionogi 13
16	16.4		*D16Ncvs14*, DNA segment, Chr 16, National Cardiovascular Center, Shionogi 14
16	16.4		*D16Ncvs15*, DNA segment, Chr 16, National Cardiovascular Center, Shionogi 15
16	16.4		*D16Ncvs16*, DNA segment, Chr 16, National Cardiovascular Center, Shionogi 16
16	16.4		*D16Ncvs17*, DNA segment, Chr 16, National Cardiovascular Center, Shionogi 17

Chromosome	*cM Position*	*Band*	*Symbol, Name*
16	16.4		*D16Ncvs18*, DNA segment, Chr 16, National Cardiovascular Center, Shionogi 18
16	16.4		*D16Ncvs19*, DNA segment, Chr 16, National Cardiovascular Center, Shionogi 19
16	16.4		*D16Ncvs2*, DNA segment, Chr 16, National Cardiovascular Center, Shionogi 2
16	16.4		*D16Ncvs3*, DNA segment, Chr 16, National Cardiovascular Center, Shionogi 3
16	16.4		*D16Ncvs4*, DNA segment, Chr 16, National Cardiovascular Center, Shionogi 4
16	16.4		*D16Ncvs5*, DNA segment, Chr 16, National Cardiovascular Center, Shionogi 5
16	16.4		*D16Ncvs7*, DNA segment, Chr 16, National Cardiovascular Center, Shionogi 7
16	16.4		*D16Ncvs8*, DNA segment, Chr 16, National Cardiovascular Center, Shionogi 8
16	16.4		*D16Ncvs9*, DNA segment, Chr 16, National Cardiovascular Center, Shionogi 9
16	16.5		*D16Xrf396*, DNA segment, Chr 16, XREFdb 396
16	16.85		*D16Sel3*, DNA segment, Chr 16, Seldin 3
16	16.9		*D16Mit146*, DNA segment, Chr 16, Massachusetts Institute of Technology 146
16	17.0		*D16Mit101*, DNA segment, Chr 16, Massachusetts Institute of Technology 101
16	17.9		*Loh2*, loss of heterozygosity, region 2
16	18.0		*D16Bir6*, DNA segment, Chr 16, Birkenmeier 6
16	18.95		*Hmg1-rs7*, high mobility group protein, related sequence 7
16	19.0		*D16Mit102*, DNA segment, Chr 16, Massachusetts Institute of Technology 102
16	19.0	cen-C3	*Smst*, somatostatin

Table 4.2 Genes in the vicinity to human AHSG

Chromosome	*Symbol*	*Name*
3q22-23	RASA2, GAP1M	RAS p21 protein activator2
3q22-24	FRP1	FRAP-related protein 1
3q25	IGKJRB1	Immunoglobulin kappa J region recombination signal binding protein
3q25.1	MLF1	Myeloid leukemia factor-1
3q25-q25.2	PFN-2,PFL,D3S1319E	Profilin-2
3q25-q26	SI	Sucroase-isomaltase
3q26	EVI1	Ectropic viral integration site-1, oncogene EVI1
3q26	MDS1	Myelodysplasia syndrome-1
3q26	RPL22,EAP	Ribosomal protein L22
3q26-qter	CLCN2	Chloride channel 2
3q26.1-q26.2	BCHE.CHE1	Butyrylcholinesterase
3q26.1-q26.2	ECT2	Epithelial cell transforming sequence 2 oncogene
3q26.1-q26.3	SLC2A2,GLUT2	Solute carrier family 2 (facilitated glucose transporter), member 2
3q26.2	TFRC	Transferrin receptor
3q26.2-q26.3	OSP	Oligodendrocyte-specific protein
3q26.2-qter	APOD	Apolipoprotein D
3q26.3	CDL	Cornelia de Lange syndrome
3q26.3	PIK3CA	Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide
3q26.3-q27	SOX2	SRY (sex determining region Y) box2
3q26.3-q27	MGDF,THPO,MLLP,TPO	Megakaryocyte growth and development factor
3q27	**AHSG**	**Alpha-2HS-glycoprotein**
3q27	BCL6	B-cell CLL/lymphoma-6
3q27	DVL3	Dishevelled 3 (homologous to Drosophila dsh)
3q27	EHHADH,PBFE	Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase
3q27	EIF4G	Eukaryotic translation initiation factor 4 gamma
3q27	FIM3	Friend murine leukemia virus integration site 3
3q27	HRG	Histidine-rich glycoprotein
3q27	KNG	Kininogen
3q27	RFC4	Replication factor C (activator 1) 4 (37 kD)
3q27-q28	CRARF	C4/C2 activating component of Ra-reactive factor
3q28	CLAPM1	Clathrin-associated/assembly/adaptor protein, medium 1

Chromosome	*Symbol*	*Name*
3q28	ETV 5,ERM	Ets variant gene 5 (ets-related molecule)
3q28	FGF12,FHF1	Fibroblast growth factor 12
3q28	LPP	Lipoma-preferred-partner gene
3q28	SST	Somatostatin
3q28-q29	HRY	Hairy (Drosophila)-homolog
3q28-q29	OPA1	Optic atrophy 1 (autosomal dominant)
3q29	DLG1	Discs, large (Drosophila) homolog
3q29	MFI2,MAP97	Melanoma-associated antigen p97
3q29	MUC4	Mucin4, tracheobronchial
3q29	PPP1R2,IPP2	Protein phosphatase1, regulatory (inhibitor) subunit 2

CHAPTER V

Functional studies of recombinant mouse α_2-HSG with respect to the insulin receptor

Introduction

We previously reported (Srinivas *et al.*, 1993) that recombinant human α_2-HSG inhibited both the insulin stimulated autophosphorylation of the insulin receptor and tyrosine kinase activity (TKA). In order to investigate whether the mouse recombinant homolog α_2-HSG also inhibits the insulin stimulated insulin receptor autophosphorylation and TKA, a series of functional studies were performed.

Materials and Methods

In vitro assay of the insulin receptor autophosphorylation

Insulin receptors were partially purified from the H-35 rat hepatoma cell line (ATCC, Rockville, MD) as described earlier (Freidenberg *et al.*, 1985). Autophosphorylation of crude insulin receptors was asessed by preincubating various concentrations of α_2-HSG in the presence of insulin (100 nM) for 30 min at 20°C. The phosphorylation reaction was initiated in the presence of [^{32}P]-ATP (3000 Ci/mmol), $MnCl_2$ (8 mM), ATP (10 μM), PNPP (10 mM), and Na-orthovanadate (100 μM). Reactions were stopped after 10 min by boiling in the presence of 3% SDS and 100 mM DTT. Proteins were separated by SDS-PAGE 10% gel and the ^{32}P incorporated wasdetected by autoradiography of the dried gel.

Insulin receptor tyrosine kinase activity

To assess IR-TK activity, partially purified receptors were incubated in the presence of insulin (100 nM), with or without α_2-HSG. The receptors were subsequently phosphorylated using [γ-^{32}P]-ATP in the presence of $MgCl_2$ (16 mM) and ATP (4 μM). An exogenous substrate, poly ($Glu^{80}Tyr^{20}$), was used to assess the IR-TK activity.

[^{3}H]-thymidine uptake during DNA synthesis

Insulin-induced DNA synthesis was monitored by incorporation of [^{3}H]-thymidine into H-35 cells. The cells were grown to 30% confluence and incubated in serum-free DMEM, containing 0.1% insulin-free BSA for 36 hours and reincubated for 14 hours with 100 nM insulin, in the presence of various concentrations of mouse α_2-HSG. Cells were pulsed with 1 μCi/ml of [^{3}H]-thymidine (NEN-Dupont, Wilmington, DE) for 1 hours, and then washed twice with ice-cold PBS. The cells were solubilized and the DNA precipitated with ice-cold TCA. The precipitates were collected on glass tubes and washed twice with ice-cold 5% TCA. The radioactivity incorporated was quantitated in a scintillation counter.

Results

In order to perform several functional studies, insulin receptors were partially purified from rat hepatoma cell line and tested for IR-TKA (Fig 5.1). The tyrosine kinase assay indicated that insulin receptor elution 3 had the highest activity (Fig. 5.1). This optimal eluate was used for the following experiments.

Mouse α_2-HSG was expressed using the baculovirus system and its identity was

verified by immunoblotting against pp63: the rat homolog. Two bands appeared prominent at 60 and 66 kD (Fig.2.11). A time course protein expression experiment was performed to identify the correlation between mouse α_2-HSG production and its tyrosine kinase activity. Sf-9 insect cells were infected with the recombinant protein α_2-HSG and aliquots were removed at time 0, 14, 24, 40, 48, 64 and 72 hours. A total of 100 μg/ml of crude recombinant supernatant was used to perform a tyrosine kinase assay. Stimulated insulin receptors were inhibited about 80% at 72 hour (Fig 5.2). The maximun expression of mouse α_2-HSG correlates with the highest activity of the protein able to inhibit the insulin-stimulated receptor tyrosine kinase activity.

The affinity purified recombinant mouse α_2-HSG was eluted and recovered. Eluates 1-5 were quantitated for protein concentrations, (Fig 5.3). A total of 30 μl of the mouse α_2-HSG eluates 1-5 were tested in a tyrosine kinase assay. The experiment demonstrated that eluate #3 (40 μg/ml) of purified mouse α_2-HSG inhibited 91% the insulin receptor tyrosine kinase activity. (Fig 5.4).

Jacalin lectin affinity-purified recombinant mouse α_2-HSG eluate #3 was used to test inhibitory activity of the insulin receptor in a TK assay at different protein concentrations. Mouse α_2-HSG was tested at concentrations ranging from 10 μg/ml to 20 μg/ml in the presence of insulin (100mM). Approximately 70% inhibition of IR-TK activity was observed at 15 μg/ml (Fig. 5.5).

Multiple experiments were performed in duplicate in order to verify the ability of recombinant mouse α_2-HSG to inhibit autophosphorylation of the 95 kD β-subunit of the IR. The proteins were separated on a 10% SDS-PAGE and the ^{32}P incorporated was detected by autoradiography of the dried gel. Mouse α_2-HSG at a concentration of 1

μg/ml completely abolished insulin-induced autophosphorylation of the β-subunit of partially purified rat IR (Fig. 5.6).

Various concentrations of recombinant mouse α_2-HSG were tested for their ability to inhibit insulin-induced DNA synthesis. Mouse α_2-HSG at 25-35 μg/ml completely abolished insulin-induced DNA synthesis in H-35 hepatoma cells (Fig. 5.7).

Discussion

In the present study, we have used the baculoviral system to express mouse α_2-HSG as a recombinant protein. The baculoviral protein has an apparent molecular weight of 60-66 kD. Several experiments indicated that optimal time point of recombinant mouse α_2-HSG expression (72 hours) represented the maximal tyrosine kinase activity inhibition of insulin stimulated receptors. It has also been shown that mouse α_2-HSG blocked insulin-induced IR-autophosphorylation at 1 μg/ml, IR-TKA at 15 μg/ml and insulin stimulated mitogenesis at 25 μg/ml *in vivo*.

In the rat, it has been shown that pp63/fetuin can inhibit IR-TK and IR autophosphorylation with a half-maximal inhibition of 0.24 μg/ml (Auberger *et al.*, 1989). These results demonstrated that the IR inhibitory activity of rat fetuin (Auberger et al., 1989), human α_2-HSG (Srinivas *et al.*, 1993; Srinivas *et al.*, 1995), and bovine fetuin (Mathews *et al.*, 1997) now extends to mouse α_2-HSG.

All these results suggest that the mouse Ahsg gene is the ortholog of the human AHSG gene. Additional studies will be necessary to determine whether the *in vitro* demonstration of IR inhibitory activity demonstrated now for rat, bovine, human and mouse proteins has a physiological significance for glucose regulation in these species.

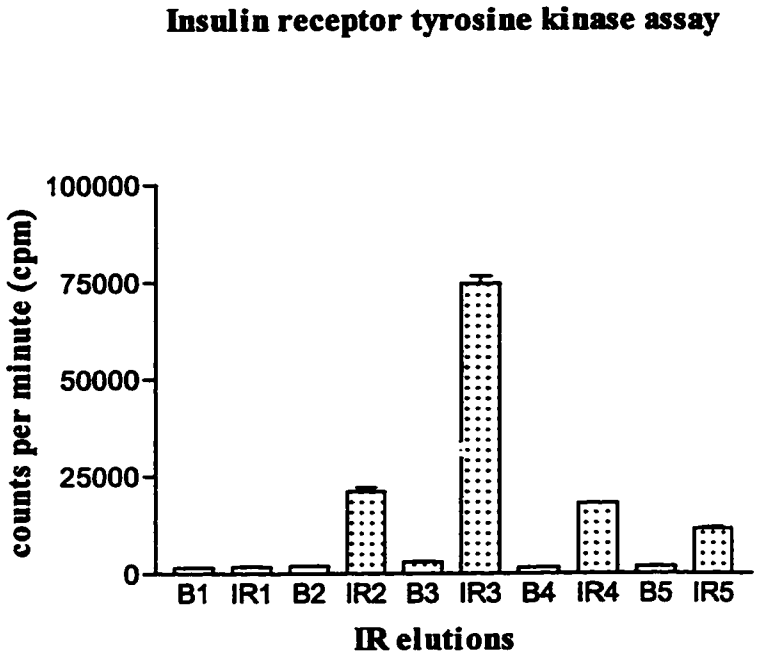


Fig. 5.1 Insulin receptor tyrosine kinase activity. Insulin receptors were partially purified from hepatoma cells, using WGA-affinity column and the eluates were collected. Eluates 1-5 were used in a tyrosine kinase assay. Eluate #3 has the highest insulin stimulated tyrosine kinase activity.

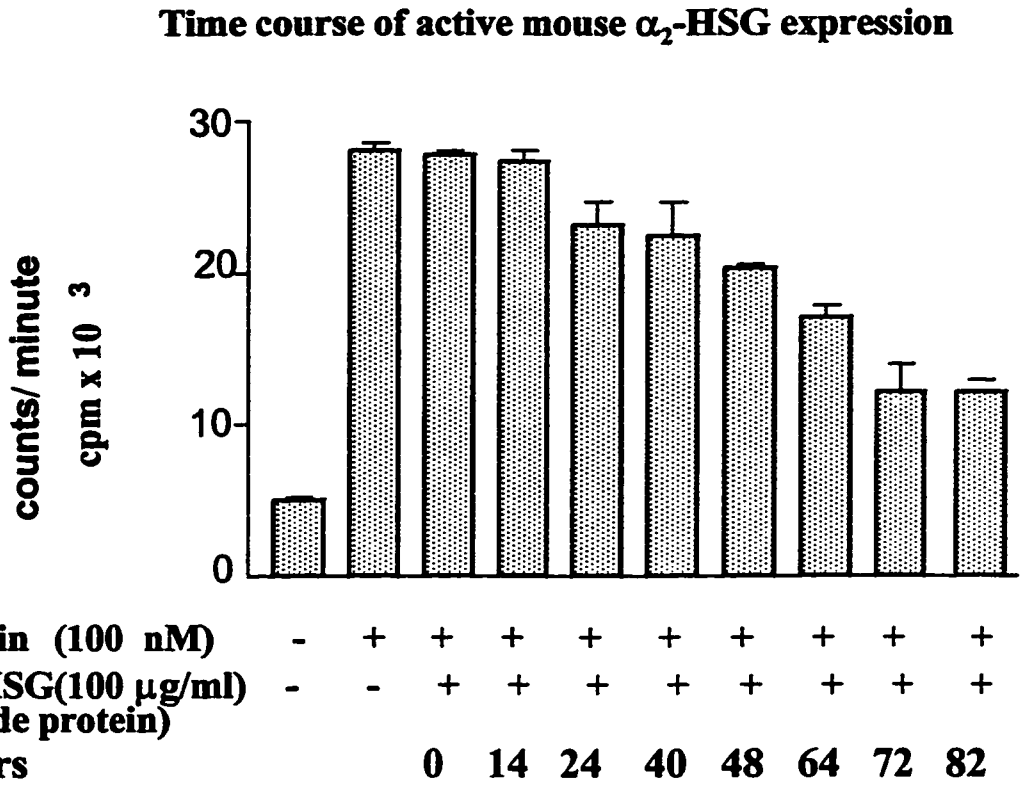


Fig. 5.2 Time course expression of recombinant mouse α_2-HSG: tyrosine kinase activity. Mouse α_2-HSG aliquots were recovered at 0, 14, 24, 40, 48, 64 and 72 hours of expression. A total of 100 µg/ml of the crude infected supernatant were used in a tyrosine kinase assay in order to identify the optimal time point of the protein expression that provided the maximal inhibition of the insulin receptor tyrosine kinase activity. The optimal point of expression of mouse α_2-HSG that provided the maximal inhibition (80%) of the insulin receptor tyrosine kinase activity was 72 hours.

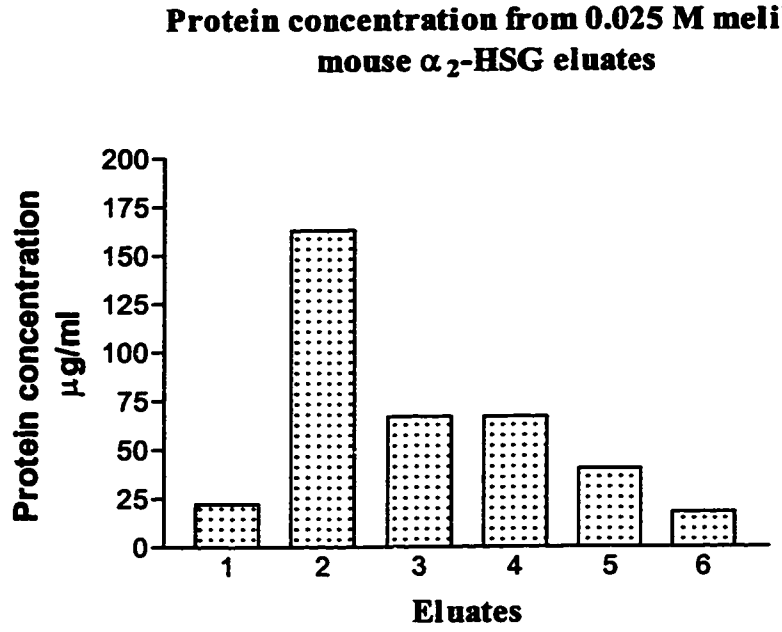


Fig. 5.3 Protein concentration of mouse α_2-HSG: eluates 1-6. Recombinant mouse α_2-HSG was purified using jacalin column and eluted with 0.025 M melibiose. Eluates 1-6 were collected and the protein concentration was measured.

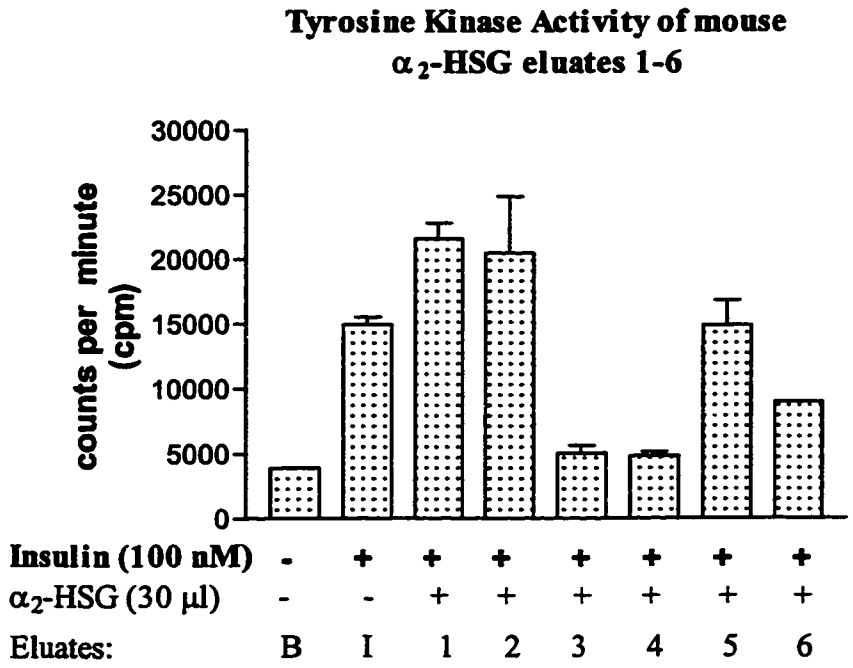


Fig. 5.4 Tyrosine kinase activity of mouse α_2-HSG eluates 1-6. Mouse α_2-HSG eluates 1-6 were used in a tyrosine kinase assay. Eluates 3 and 4 inhibited 91% of the tyrosine kianse activity of the insulin-stimulated IR. B: Basal, I: Insulin.

Fig. 5.5 Recombinant mouse α_2-HSG is an inhibitor of the insulin receptor (IR) at the tyrosine kinase (TK) level. The bar graph indicates that the tyrosine kinase activity of the insulin receptor is inhibited by α_2-HSG. IRs purified from H-35 rat hepatoma cells were incubated for 30 min, 20°C with various concentrations of mouse α_2-HSG (10, 15, or 20 μg/ml) in the presence or absence of insulin (100 nM). Insulin-stimulated IR-TKA was assessed by its ability to transfer ^{32}P onto the synthetic substrate poly ($Glu^{80}Tyr^{20}$). Lane 1 represents stimulation of the insulin receptor with 100 nM insulin. Mouse α_2-HSG inhibits over 70% the insulin stimulated receptors at a concentration of 15 μg/ml. The results shown are representative of three separate experiments.

Effect of α_2-HSG on TK activity of insulin receptors *in vitro*

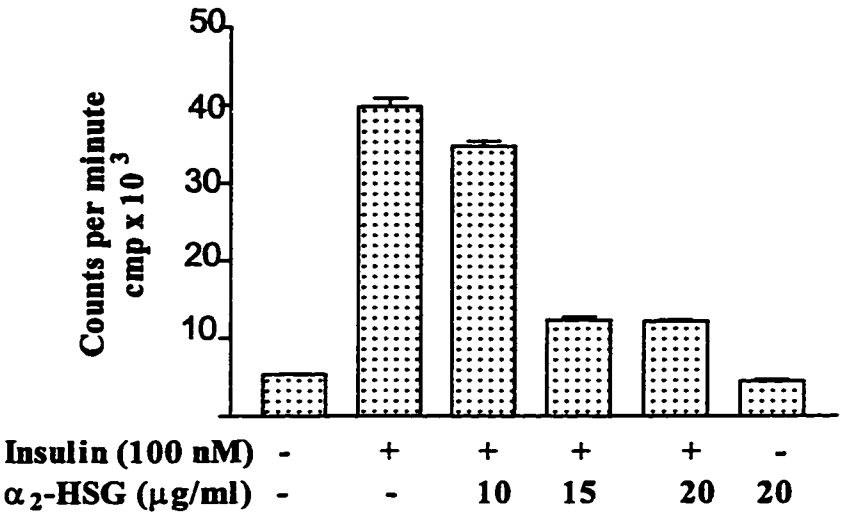


Fig. 5.6 Effect of α_2-HSG on autophosphorylation of the IR *in vitro*. The autoradiograph reveals that recombinant mouse α_2-HSG inhibits the autophosphorylation of the insulin receptor. Partially purified insulin receptors from H-35 cells were pre-incubated with or without insulin (100 nM) in presence or absence of mouse α_2-HSG (1, 10, 75 μg/ml) respectively. Autophosphorylation was determined as described in Materials and Methods. The position of the 95 kDa β-subunit of the IR is indicated. Insulin-induced autophosphorylation of the β-subunit of the IR compared to basal (lanes 2 and 1, respectively). Purified mouse protein at concentrations of 1 μg/ml completely abolished insulin-induced autophosphorylation of the IR.

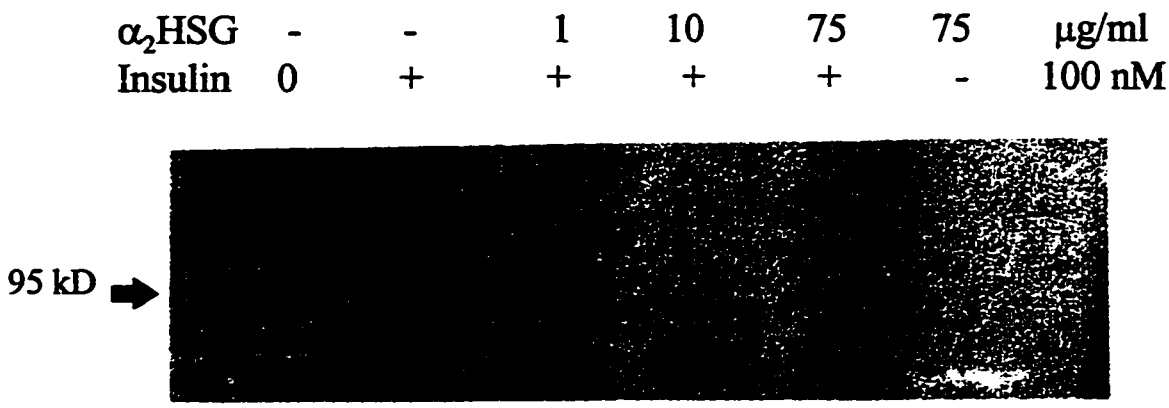
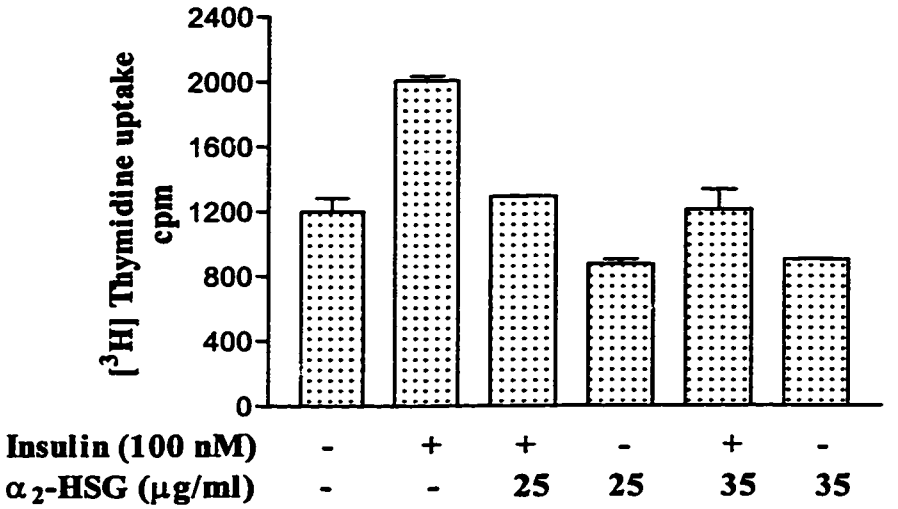

α2HSG - - 1 10 75 75 μg/ml
Insulin 0 + + + + - 100 nM
95 kD

Fig. 5.7 Mouse α_2-HSG inhibits insulin-induced mitogenesis on H-35 hepatoma cells. The data describe the effect of recombinant mouse α_2-HSG on insulin-induced mitogenesis of H-35 rat hepatoma cells. Subconfluent dishes were treated as described in Materials and Methods and the uptake of [^{3}H]-thymidine was measured. Insulin induced DNA synthesis (represented in the first bar). Insulin-stimulated mitogenesis is completely blocked at concentrations of 25-35 μg/ml of mouse α_2-HSG (bar 2).

Effect of α_2-HSG on insulin-induced mitogenesis of H-35 hepatoma cells



References

Akhoundi, C., Amiot, M., Auberger, P., Le Cam, A., Rossi, B. (1994). Insulin and interleukin-1 differentially regulate pp63, an acute phase phosphoprotein in hepatoma cell line. *J. Biol.Chem.* **269**: 15925-15930.

Araki, T., Yoshioka, Y., Schmid, K. (1989). The position of the disulfide bonds in human plasma α_2HSG and the repeating double disulfide bonds in the domain structure. *Biochim. Biophys. Acta.* **994**: 195-199.

Ashton, B. A., Höhling H-J., Triffitt, J. T. (1976). Plasma proteins present in human cortical bone: enrichment of the α_2-HS-glycoprotein. *Calcif.Tissue. Res.* **22**: 27-33.

Ashton B. A., Smith R. (1980). Plasma α_2-HS-glycoprotein concentration in Paget's disease of bone: its possible significance. *Clin. Sci.* **58**: 435-438.

Auberger, P., Falquerho, L., Contreres J.O., Pages, G., Le Cam, G., Rosii, B., Le Cam, A. (1989). Characterization of a natural inhibitor of the insulin receptor tyrosine kinase: cDNA cloning, purification, and anti-mitogenic activity. *Cell* **58**: 631-640.

Banine F, Gangneux C, Lebreton JP, Frebourg T, Salier JP. (1998). Structural and functional analysis of the 5'-transcription control region for the human α2-HS glycoprotein gene. *Biochim. Biophys. Acta* **1398**(1), 1-8.

Brown, W.M., Dziegielewska, K.M., Saunders, N.R., Christie, D.L., Nawratil, P., Müller-Esterl, W. 1992). The nucleotide and deduced amino acid structures of sheep and pig fetuin: common structural features of the mammalian fetuin family. *Eur. J. Biochem.* **205**: 321-331.

Christie, D. L., Dziegielewska, K. M., Hill, R. M., Saunders, N. R. (1987). Fetuin: the bovine homologue of human alpha 2HSglycoprotein. *FEBS Letters.* **214**: 45-49.

Dickson, I. R., Poole, A. R. and Veis, A. (1975). Localization of plasma α_2HS-glycoprotein in mineralising human bone. *Nature* **256**, 430-432.

Dziegielewska, K.M., Mollgard, K., Reynolds, M.L., Saunders, N.R. (1987). A fetuin-related glycoprotein (α2HS) in human embryonic and fetal development. *Cell Tissue Res* **248**(1):33-41.

Dziegielewska, K.M., Brown, W. N., Casey, S.J.,Christie, D. L., Foreman, R.C., Hill, R. M., Saunders, N. R. (1990). The complete cDNA and amino acid sequence of bovine fetuin. Its homology with alpha 2HS glycoprotein and relation to other members of the cystatin superfamily. *J. Biol. Chem.* **265**: 4354-4357.

Elzanowski, A., Barker, W.C., Hunt, L. T., Seibel-Ross E. (1988) Cystatin domains in alpha-2-HS-glycoprotein and fetuin. *FEBS Letters.* **227**: 167-170.

Falquerho, L., Patey, G., Paquereau, L., Rossi, V., Lahuna, O., Szpirer, J., Szpirer, C., Lavan, G., Le Cam, A. (1991). Primary structure of the rat gene encoding an inhibitor of the insulin receptor tyrosine kinase. *Gene.* **98**: 209-216.

Falquerho, L., Paquereau, L., Vilarem, M.J., Galas, S., Patey, G., Le Cam, A. (1992). Functional characterization of the promoter of pp63, a gene encoding a natural inhibitor of the insulin receptor tyrosine kinase. *Nucleic Acids Res.* **20**(8): 1983-1990.

Freidenberg, G.R., Klein, H. H., Cordera, R., Olefsky, J.M. (1985). Insulin receptor kinase activity in rat liver. *J.Biol.Chem.* **260**: 12444-12453.

Haasemann, M., Nawratil, P., Müller-Esterl, W. (1991). Rat tyrosine kinase inhibitor shows sequence similarity to human α_2HSG and bovine fetuin. *Biochem. J.* **274**: 899-902.

Hani, E.H., Suaud, L., Boutin, P., Chevre, J.C., Durand, E., Philippi, A., Demenais, F.,Vionnet, N.,Furuta, H., Velho, G., Bell, G.I., Laine, B., Froguel, P. (1998). A missense mutation in hepatocyte nuclear factor-4alpha, resulting in a reduced transactivation activity, in human late-onset non-insulin-dependent diabetes mellitus. *J. Clin. Invest.* **101**: 521-526.

Hayase, T., Rice K.G., Dziegielewska, K. M., Kuhlenschmidt, M., Reilly, T., Lee, Y.C.(1992). Comparison of N-glycosides of fetuins from different species and human alpha 2-HS-glycoprotein. *Biochemistry.* **31**: 4915-4921.

Heremans J. F. (1960). Les Globulines Seriques du Système Gamma, pp103-107, Arcia, Brussels.

Kaisaki, P.J., Menzel S., Lindner, T., Oda, N.,Rjasanowski, I., Sahm, J., Meincke, G., Schultze, J., Schmechel, H., Petzold, C., Ledermann, H. M., Sachse, G., Boriraj, V.V., Menzel, R.,Kerner, W., Turner, R.C., Yamagata, K., Bell, G.I. (1997). Mutations in the hepatocyte nuclear factor-1alpha gene in MODY and early-onset NIDDM:evidence for a mutational hotspot in exon 4. *Diabetes* **46**: 528-535.

Jahnen-Dechent, W., Trindle, A., Godovac-Zimmerman, J., Müller-Esterl. W. (1994). Postranslational modification processing of human α_2HSG: evidence for the production of a phosphorylated single-chain form by hepatoma cells. *Eur. J. Biochem.* **226**: 59-69.

Jahnen-Dechent, W., Schinke, T., Trindl, A., Müller-Esterl, W., Sablitzky, F., Kaiser, S., Blessing, M. (1997). Cloning and targeted deletion of the mouse fetuin gene. *J. Biol. Chem.* **272**(50), 31496-31503.

Kalabay, L., Cseh, K., Benedek, S., Fakete, S., Masszi, T., Herjeczki, K., Pozsonyi, T., Jakab, L., Jakab, L. (1991). Serum alpha 2-HS glycoprotein concentration in patients with hematological malignancies. A follow-up study. *Ann Hematol.* **63**:

264-269.

Keely, F. and Sitarz, E. E. (1985). Characterization of proteins from the calcified matrix of atherosclerotic human aorta. *Atherosclerosis* **46**: 29-40.

Kellerman, J., Haupt, H., Auerswald, E.A., and Müller-Ester, W. (1989). The arrangement of disulfide loops in human α_2-HS glycoprotein. Similarity to the disulfide bridge structures of cystatins and kininogens. *J. Biol. Chem.* **264**: 14121-14128.

King, D., Snider, L.D., Lingrel, J.B. (1986). Polymorphism in an androgen-regulated mouse gene is the result of the insertion of a B1 repetitive element into the transcription unit. *Mol. Cell. Biol.* **6**: 209-217.

Ko, M. S. H., Wang, X., Horton, J. H., Hagen, M. D., Takahashi, N., Maezaki, Y. and Nadeau, J. H. (1994). Genetic mapping of 40 cDNA clones on the mouse genome by PCR. *Mamm. Genome* **5**: 349-355.

Koide, T.(1988). Human histidine-rich glycoprotein gene: evidence for evolutionary relatedness to cystatin supergene family. *Thromb.Res.Suppl.* **8**: 91-97.

Labuda, D., Sinnett, D., Richer, C., Deragon, J.-M., Striker, G. (1991). Evolution of mouse B1 repeats: 7SL RNA folding pattern conserved. *J. Mol. Evol.* **32**: 405-414.

Lebreton J. P., Joisel F., Raoult J. P., Lannuzel B., Rogez J. P. and Humbert G. (1979). Serum concentration of human α_2-HS glycoprotein during the inflammatory process. *J. Clin. Invest.* **64**: 1118-1129.

Le Cam, A., Magnaldo, I., Le Cam, G., Auberger, P. (1985). Secretion of major phosphorylated glycoprotein by hepatocytes. Characterization of specific antibodies and investigation of the processing, excretion kinetics, and phosphorylation. *J. Biol.Chem.* **260**: 15965-15971.

Le Cam, A., Le Cam, G. (1985). A new negatively regulated acute phase phosphoprotein synthesized by rat hepatocytes. *Biochem. J.* **230**: 603-607.

Le Cam, A., Auberger, P. Falquerho, L., Contreres, J.O., Pages, G., Le Cam, G., Ross, B. (1992). pp63 is very likely the rat fetuin (letter). *Cell* **68**: 8.

Lee, C-C., Bowman, B.H., Yang, F.(1987) Human α_2HSG: The A and B chains with connecting sequence are encoded by a single mRNA transcript. *PNAS USA.* **84**: 4403-4407.

Levitt, R.C. (1991) Polymorphisms in the transcribed 3' untranslated region of eukaryotic genes. *Genomics* **11**: 484-489.

Lewis, J. G., and Andre, C. M. (1980). Effect of human α_2HS-glycoprotein on mouse

macrophage function. *Immunology* **39**: 317-322.

Lewis, J. G., and Andre, C. M. (1981). Enhancement of human monocyte phagocytic function by α_2-HS-glycoprotein. *Immunology* **42**: 481-487.

Lewis J. G., PhD. thesis, pp 1-1885 (1983). University of Otago, Dunedin, New Zealand.

Lindner, T., Gragnoli, C., Furuta, H., Cockburn, B. N., Petzold, C., Rietzsch, H., Weiss, U., Schulze, J., Bell, G.I. (1997). Hepatic function in a family with a nonsense mutation (R154X) in the hepatocyte nuclear factor-4 alpha/MODY1 gene. *J.Clin Invest.* **100**: 1400-1405.

Lowe, T., Sharefkin, J., Yang, S.Q., Dieffenbach, C.W. (1990). A computer program for selection of oligonucleotides primers for polymerase chain reactions. *Nucleic Acids. Res.* **18**: 1757-1761.

Malone,J.D., Teitelbaum, S. L., Griffin, G.L., Senior, R. M. and Kahn, A. J. (1982). Recruitment of osteoclast presursors by purified bone matrix constituents. *J. Cell Biol.* **92**: 227-230.

Manly, K. F. (1993). A Macintosh program for storage and analysis of experimental genetic mapping data. *Mamm. Genome* **4**: 303-313.

Mathews, S.T., Srinivas, P.R., Leon, M.A., Grunberger, G. (1997). Bovine fetuin is an inhibitor of insulin receptor tyrosine kinase. *Life Sci.* **61**: 1583-1592.

Mbuyi, J.-M., Dequeker, J., Bloemmen, F. and Stevens, E. (1982). Plasma proteins in human cortical bone: enrichment of α_2HS-glycoprotein, α1 acid-glycoprotein, and IgE. *Calcif. Tissue. Int.* **34**: 229-231.

Olesfky, J.M. (1990). The insulin receptor: a multifunctional protein. *Diabetes* **39**: 1009-1016.

Osawa, M., Umetsu, K., Sato, M., Ohki, T., Yukawa, N., Suzuki, T., Takeichi, S. (1997). Structure of the gene encoding human alpha 2-HS glycoprotein (AHSG). *Gene.* **196**: 121-125.

Poduslo, S.E., Dean, M., Kolch, U., O'Brien, S.J. (1991). Detecting high-resolution polymorphisms in human coding loci by combining PCR and single-stranded conformation polymorphism (SSCP). *Am. J. Hum. Genet.* **49**: 106-111.

Quelch, K. J., Cole, W. G. and Melick, R. A. (1984). Noncollagenous proteins in normal and pathological human bone. *Calcif. Tissue. Int.* **36**: 545-549.

Quentin, Y. (1989). Successive waves of fixation of B1 variants in rodent lineage history. *J. Mol. Evol.* **28**: 299-305.

Rauth, G., Pöschke, O., Fink, E., Eulitz, M., Tippmer, S., Kellerer, M., Haring, H-U,

Nawratil, P.,Haasemann, M., Jahnen-Dechent, W., Müller-Esterl, W. (1992). The nucleotide and partial amino acid sequences of rat fetuin: identity with the natural tyrosine kinase inhibitor of the rat insulin receptor. *Eur. J. B iochem.* **204**:52 529.

Rizzu, P., Baldini, A. (1995). Three members of human cystatin gene superfamily, AHSG,HRG,and KNG, map within one megabase of genomic DNA at 3q27. *Cytogent. Cell. Genet.* **70**: 26-28.

Rowe, L. B., Nadeau, J. H., Turner, R., Frankel, W. N., Letts, V. A., Eppig, J. T., Ko, M. S. H., Thurston, S. J. and Birkenmeier, E. H. (1994). Maps from two interspecific backcross DNA panels available as a community genetic mapping resource. *Mamm. Genome* **5**: 253-274.

Saksela, K., Baltimore, D.(1993). Negative regulation of immunoglobin kappa light chain gene transcription by a short sequence homologous to the murine B1 repetitive element. *Mol. Cell. Biol.* **193**: 3698-3705.

Schinke, T., Amendt, C., Trindl, A., Pöschke, O., Müller-Esterl, W., Jahnen-Dechent, W. (1996). The serum protein α_2-HS glycoprotein/fetuin inhibits apatite formation in vitro and in mineralizing calvaria cells. *J. Biol. Chem.* **271**: 20789-20796.

Schmid K. and Burgi, W. (1961). Preparation and proliferation of the human plasma Ba-α2-HS glycoprotein. *Biochim. Biophys. Acta* **47**:440-453.

Srinivas, P.R., Wagner, A.S., Reddy, L.V., Deutsch, D.D., Leon, M.A., Goustin, A.S., Grunberger, G. (1993). Serum α_2HSG is an inhibitor of the human insulin receptor at the tyrosine kinase level. *Mol.Endo.* **7**: 1445-1455.

Srinivas, P.R., Goustin, A.S., Grunberger, G. (1995). Baculoviral expression of a natural inhibitor of the human insulin receptor tyrosine kinase. *Biochem. Biophys. Res. Commun.* **208**: 879-885.

Srinivas P.R.,Deutsch, D.D., Mathews, S.T., Goustin, A.S., Leon, M.A., Grunberger, G. (1996). Recombinant human α_2-HS glycoprotein inhibits insulin-stimulated mitogenic pathway without affecting metabolic signaling in Chinese Hamster Ovary cells overexpressing the human insulin receptor. *Cell. Signal.* **8**: 567-573.

Takagaki, Y., Kitamura, N., Nakanishi, S. (1985). Cloning and sequence analysis of cDNAs for human high molecular weight and low molecular weight prekininogens. Primary structures of two human prekininogens. *J. Biol.Chem.* **260**: 8601-8609.

Takahashi, N. and Ko, M. S. H. (1993). The short 3'-end region of complementary DNAs as PCR-based polymorphic markers for an expression map of the mouse genome. *Genomics* **16**: 161-168.

Takahashi, N.,Ko, M.S. (1994). Toward a whole cDNA catalog: construction of an

equalized cDNA library from mouse embryos. *Genomics* **23**: 202-210.

Terkelsen OBF, Jahnen-Dechent W, Nielsen H, Moos T., Fink, E., Nawratil, P., Müller-Esterl, W., Mollgard, K. (1998). Rat fetuin: distribution of protein and messenger RNA in embryonic and neonatal rat tissues. *Anatomy and Embryology* **197(2)**, 125-133.

Triffitt, J. T., Gebauer, U., Ashton, B.A., Owen, M.E. and Reynolds, J. J. (1976). Origin of plasma α_2HS-glycoprotein and its accumulation in bone. *Nature* **262**: 226-227.

Van Obberghen, E. (1994). Signaling through the insulin receptor and the insulin-like growth factor-1 receptor. *Diabetologia* **37** [Suppl]: S125-S134.

Van Oss, C. J., Gillman, C. F., Bronson, P. M. and Border, J. R. (1974). Opsonic properties of human serum alpha $_2$ HS- glycoprotein. *Immunol. Commun.* **3**: 329-335.

Winoto, A., Baltimore, D., (1989). αβ Lineage-specific expression of the αT cell receptor gene by nearby silencers. *Cell* **59**: 649-655.

Yang, F., Chen, Z.L.,Bergeron, J.M., Cupples, R.L., Friedrichs, W.E. (1992). Human alpha 2-HS-glycoprotein/bovine fetuin homologue in mice: identification and developmental regulation of the gene. *Biochim. Biophys. Acta* **1130**: 149-156.

Yoshioda, Y., Gejyo, F., Marti, T., Rickli, E.E., Bürgi, W., Offner, G.D., Troxler, R.F., Schmid, K. (1986). The complete amino acid sequence of the A-chain of human plasma α_2HS-glycoprotein. *J. Biol.Chem.* **261**: 1665-1676.

ABSTRACT

GENETIC MAPPING AND FUNCTIONAL STUDIES OF A NATURAL INHIBITOR OF THE INSULIN RECEPTOR TYROSINE KINASE: THE MOUSE ORTHOLOG OF HUMAN α_2-HS GLYCOPROTEIN

by

VIVIAN J. CINTRON

December 1998

Co-Advisors: Dr. George Grunberger
Dr. Hiroshi Mizukami

Major: Biological Sciences (Molecular Biology)

Degree: Doctor of Philosophy

Fetuin/α_2-HS glycoprotein (α_2-HSG) homologs have been identified in several species including rat, sheep, pig, rabbit, guinea pig, cattle, mouse and human. Multiple physiological roles for these homologs have been suggested, including ability to bind to hydroxyapatite crystals and to specifically inhibit the tyrosine kinase (TK) activity of the insulin receptor (IR). In this study we report the identification, cloning, and characterization of the mouse Ahsg gene and its function as an IR-TK inhibitor. Genomic clones derived from a mouse Svj 129 genomic library were sequenced in order to characterize the intron-exon organization of the mouse Ahsg gene, including an 875 bp subclone containing 154 bp upstream from the transcription start site, the first exon, and part of the first intron. A second genomic subclone harboring a 3.45 kb BgI II fragment contained exons 2, 3, and 4 in addition to two adjacent elements within the first intron--a repetitive element of the B1 family (92 bp) and a 271 bp tract of (T,C)n *(A,G)n. We have mapped mouse Ahsg at 16 cM adjacent to the Dagk3 gene on chromosome 16 by genotyping interspecific backcross panels between C57BL/6J and *Mus spretus*. The

position is syntenic with human chromosome 3q27, where the human AHSG gene resides. Using recombinant mouse α_2-HSG expressed from a recombinant baculovirus, we demonstrate that mouse α_2-HSG inhibits insulin-stimulated IR autophosphorylation and IR-TKA *in vitro*. In addition, mouse α_2-HSG (25 μg/ml) completely abolishes insulin-induced DNA synthesis in H-35 rat hepatoma cells. Based on the sequence data and functional analysis, we conclude that the mouse Ahsg gene is the true ortholog of the human AHSG gene.

AUTOBIOGRAPHICAL STATEMENT

VIVIAN J. CINTRON

ADDRESS

Home: 5440 Cass Avenue Apt. 711
Detroit, MI 48202
(313) 832-1405

Office: 420 East Canfield
Elliman Building 2109
Department of Endocrinology
Wayne State University
Detroit, MI 48201
313-577-1082
Fax: 313-577-2533
vcintron@cmb.biosci.wayne.edu

EDUCATION

1998	*Doctor of Philosophy* Biological Sicences (Molecular Biology).Wayne State University (WSU), Detroit, MI.
1991	*Baccalaureate of Science* Biology, Department of Biology, University of Puerto Rico, Mayaguez, PR.

FELLOWSHIPS AND AWARDS

1991-96	*Doctoral Fellowship.*Wayne State University Liberal Arts and Science Doctoral Fellowship, Detroit, MI.
1996-1998	*Predoctoral Fellowship- National Institutes of Health.* National Institute of Diabetes and Digestive and Kidney Diseases. Grant #5 F31 DK09521.
1997-1998	*Blue Cross Blue Shield of Michigan Foundation Student Award* Grant #241-SAP/97.

PUBLICATIONS

Vivian J. Cintron, Minoru S. H., Pothur R. Srinivas, Anton S. Goustin and George Grunberger. **Genetic Mapping and Functional Studies of a Natural Inhibitor of the Insulin Receptor Tyrosine Kinase Activity: The Mouse Ortholog of Human α_2-HS Glycoprotein** (Submitted).

Suresh T. Mathews, Nisha Chellam, Pothur. R. Srinivas, **Vivian J. Cintron**, Myron A. Leon, Anton S. Goustin and George Grunberger. **α_2-HS Glycoprotein (human fetuin) Inhibits Insulin Receptor Autophosphorylation in Intact cells and *in vivo*: Reversibility, Specificity and Inhibition of Trypsin-activated Insulin Receptor** (Submitted).

DATE AND PLACE OF BIRTH: December 7, 1968; Humacao, PR.

PROFESSIONAL MEMBERSHIPS: American Diabetes Association, American Society for Women in Science, The Endocrine Society